ISOLATION AND CHARACTERISATION OF

TEMPERATE BACTERIOPHAGES OF THE

HYPERVIRULENT *Clostridium difficile* 027 STRAINS

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Abstract

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Clostridium difficile 027 can be divided into several subclades which vary in their disease severity. Genomic studies of *C. difficile* 027 strains have established that they are rich in mobile genetic elements including prophages. Little is known about temperate bacteriophage carriage in *C. difficile* 027 clinical isolates. Therefore, this study was designed to induce and characterise temperate bacteriophages from *C. difficile* 027 subtypes' clinical isolates as a first step to understanding their potential role in disease and diversity. Ninety-one *C. difficile* 027 clinical isolates were induced for prophage, and the bacteriophages present were characterised using transmission electron microscopy and pulsed-field gel electrophoresis. A correlation between phage morphology and subtype was established. Morphological and genetically distinct tailed bacteriophages belonging to *Myoviridae* and *Siphoviridae* were identified in 62 and three isolates, respectively. Dual phage carriage was observed in four isolates. There were inducible phage tail-like particles in all the isolates. The capacity of the two antibiotics norfloxacin and mitomycin C to induce prophages was compared and it was found that they induced specific prophages from *C. difficile* isolates. PCR assays targeting the capsid, holin and portal genes of the myoviruses were designed to examine molecular diversity of *C. difficile* myoviruses. Phylogenetic analysis of the genes sequences from ten ribotypes showed that all sequences found in the ribotype 027 strains were identical and distinct from other *C. difficile* ribotypes and other bacteria species. Sporulation which can be influenced by histidine kinase gene encoded by *C. difficile* phages was characterised in 41 isolates. The isolates sporulated within the first 96 h producing between 10^4 and 10^{12} CFU/ml spores. The variation in sporulation characteristics did not correlate to the subtypes or prophage contents of the isolates examined. This study strongly suggests that phages and sporulation contribute to diversity, evolution and success of this pathogen.

Acknowledgements

List of Publications

1. **Nale, J. Y.,** Shan, J., Hickenbotham, P. T., Fawley, W. N., Wilcox, M. H. & Clokie, M. R. J. 2012. Diverse Temperate Bacteriophage Carriage in *Clostridium difficile* 027 Strains. *PLoS ONE,* 7**,** e37263.

2. Shan, J., Patel, P. V., Hickenbotham, P. T., **Nale, J. Y.,** Hargreaves, K. R. & Clokie, M. R. J. 2012. Prophage carriage and diversity within clinically relevant strains of *Clostridium difficile*. *Applied and Environmental Microbiology,* 78, 6027-34

Abbreviations

AAD: Antibiotic Associated Diarrhea

ANOVA: Analysis of variance

B1: *C. difficile* group B1 by restriction-endonuclease analysis (REA)

BHI: Brain heart infusion

BHIS: Brain heart infusion supplemented agar

BLAST: Basic local alighment search tool

Blastp: protein BLAST

Blastn: nucleotide BLAST

CaCl$_2$: Calcium chloride

CCEY: Cycloserine cefoxitin and egg yolk agar (Brazier's selective medium)

CDAD: *C. difficile* Associated Diarrhea

CDI: *Clostridium difficile* infection

CFU/ml: Colony-forming unit per milliliter

DH: Department of Health

DNA: Deoxyribonucleic acid

dsDNA: double stranded DNA

dsRNA: double stranded RNA

dATP: deoxyadenosine triphosphate

dCTP: deoxycytosine triphosphate

dGTP: deoxyguanine triphosphate

dNTPs: deoxyribonucleotide triphosphate

dTTP: deoxythymidine triphosphate

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EMBL-EBI: European Molecular Biology Laboratory-European Bioinformatics Institute

FAB: Fastidious anaerobe broth

GC: Guanine-cytosine

HPA: Health Protection Agency

IUPAC: International union of pure and applied chemistry

LB: Luria Bertani

M: mitomycin C

MgCl: Magnessium chloride

MgSO$_4$: Magnessium sulphate

MLVA: Multilocus variable-number tandem-repeat analysis

N: norfloxacin

NaCl: Sodium chloride

NAP: North American pulsed-field type

NAP1: North American pulsed-field type 1

NAP2: North American pulsed-field type 2

NCBI: National centre for biotechnology information

NCTC: National collection of type cultures

NHS: National Health Service

OD$_{550}$: (OD550 nm) Optical density at 550 nm wave length

PBS: Phosphate buffered saline

PCR: Polymerase chain length polymorphism

PEG: Polyethylene glycol

PFGE: Pulsed-field gel electrophoresis

PFU/ml: Plaque-forming unit per milliliter

PMC: Pseudomembranous colitis

PFU/ml: Plaque-forming unit per milliliter

RFLPA: Restriction Fragment Length Polymorphism Analysis

RNA: Ribonucleic acid

rpm: Revolution per minute

rRNA: ribosomal RNA

RT-PCR: Real time polymerase chain length polymorphism

SDS-PAGE: Polyacrylamide gel electrophoresis

SEM: Scanning electron microscopy

SOC: super optimal broth medium

SSC: saline-sodium citrate

ssDNA: single stranded DNA

ssRNA: single stranded RNA

TAE: Tris-acetate-EDTA

TBE: Tris-Borate-EDTA

TE: Tris-EDTA

TEM: Transmission electron microscopy

ToxA: *C. difficile* toxin A

ToxB: *C. difficile* toxin B

UPH$_2$0: Ultra pure water

UPMGA: Outweighed pair group method with arithmetic mean clustering

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1 General introduction

1.1 The *Clostridium difficile*

Clostridium difficile is the major cause of pseudomembranous colitis (PMC) and accounts for 15-39 % antibiotic associated diarrhoea (Dubberke and Wertheimer, 2009, McFarland, 2009a, Viswanathan *et al.*, 2010). *C. difficile* infection (CDI) is a worldwide health threat (Prescott *et al.*, 2002, Bartlett, 2006, Barnett, 2012). According to the HPA monthly report, there were 13,805 CDI cases in the last financial year (April 2011-March, 2012) in NHS Trusts among patients from two years and above (HPA, 2012). This figure represents 36 % reduction on the 21,675 from the previous year, 46 % on the 25,604 cases of 2009/10 and 62 % reduction on the 36,095 cases of 2008/09. Similarly, deaths involving *C. difficile* have significantly reduced with 2,710 deaths in 2010 which represents 67.5 % reduction from 2007 (HPA, 2011). Although these reports show that CDI is less frequent now in the UK, nevertheless, the number of reported as well as fatal cases are still considerable high (Al-Eidan *et al.*, 2000, Wiegand *et al.*, 2012). In addition, CDI had become more severe, and its control and treatment still remain a great problem with increased clinical and financial burden (Wilcox *et al.*, 1996, Dubberke and Wertheimer, 2009, Wiegand *et al.*, 2012). A previous study showed that CDI mortality at 30 days is 42 % in the UK and the financial burden has increased from £4,107 in 1996 to £4,500 per treatment in 2010 (Okoro *et al.*, 2008). Currently, the UK carries the highest number of 30-day mortality rate (30 %) and treatment of *C. difficile* infection currently stands at £4, 577 (Wiegand *et al.*, 2012). Reason for the high CDI morbidity and mortality is primarily due to the emergence of the epidemic *C. difficile* 027 clones and ribotypes 106, 003 and 005

(Fawley *et al.*, 2008, Dale and Gerding, 2010, Miyajima *et al.*, 2011, Wiegand *et al.*, 2012). The ribotype 027 accounts for 48 % reported cases and 11 % of fatality in 2012 which has more than doubled from the 5 % reported in 2011 (Bauer *et al.*, 2011, Wiegand *et al.*, 2012). Therefore, there is a great need to determine factors that are contributing to the diversity and pathogenicity of *C. difficile* ribotype 027 strains.

1.1.1 Morphology and taxonomy

Clostridium difficile is a Gram-positive bacterium from the genus *Clostridium*, Family: Clostridiaceae. *C. difficile* is anaerobic and readily forms endospores which can be either terminal or sub-terminal (Durre, 2005a, Merrigan *et al.*, 2010). The vegetative cell is approximately 3-5 μm in length and between 1.5 to 2 μm wide. When viewed under the microscope, the rods appear singly, in pairs or in short chains. Although *C. difficile* is predominantly Gram-positive, older colonies may exhibit cells with marked Gram variability (Beveridge, 1990, Pechine *et al.*, 2005). The Gram variability can be attributed to the fact that old and dying cells become friable due to osmotic and dehydration stress and thus become sensitive to the Gram stain (Beveridge, 1990). Colonies of *C. difficile* are morphologically variable too. Typically after 24-48 h incubation on BHI 7 % blood medium; they are 3-5 mm in diameter with irregular, lobate or rhizoidal edges. The colonies may be grey, opaque and non-haemolytic on blood agar. After a long incubation period of approximately 72 h, colonies may develop a light grey or whitish centre, a factor associated with sporulation in *C. difficile* (Aktories and Wilkins, 2000).

1.1.2 Brief history

Clostridium difficile was first reported in 1935 by Hall and O'Toole as a component of the faecal flora of healthy neonates and named *Bacillus difficilis* because of its rod shape and fastidious nature (Hall and O'Toole, 1935). These researchers were

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also the first to report that *B. difficilis* is toxigenic in hamsters, rabbits and guinea pigs (Hall and O'Toole, 1935). It was later discovered that it belonged to the class Clostridia and was then re-named *C. difficile* in the 1970s. Presumably because it was realised it fitted into the Clostridium (an obligate anaerobe) and not Bacillus (an aerobe or a facultative anaerobe). The bacterium was linked to antibiotic use following a study which showed a high percentage of patients developed PMC after treatment with clindamycin (Bartlett *et al.*, 1978a, Bartlett *et al.*, 1978b). At that time, stools of patients with pseudomembranous colitis were found to contain high levels of cytotoxic activity. The cytotoxic effects were initially attributed to viruses and mycoplasma but tests for these organisms were negative. A toxin-producing bacterium was then suspected. After extensive work on various Clostridial species, the cytotoxic activity was found to be neutralised by antiserum of *Clostridium sordelli* (Bartlett *et al.*, 1978a). As *C. difficile* was later shown to be present in more patients than those infected with *C. sordelli* and other Clostridial species, it was then concluded that *C. difficile* was the causal agent of PMC (Bartlett *et al.*, 1978a, Lyerly *et al.*, 1988). Since then, *C. difficile* has been recognised as the main etiological agent of antibacterial associated diarrhoea (AAD) and PMC, a severe necrotising and often fatal disease of the colon (Silva *et al.*, 1984, Rohner *et al.*, 1997).

1.1.3 Ecology

The distribution of *C. difficile* is widespread in the environment and it has been recognised as an important emerging gut pathogen of both humans and animals (Calfee, 2008, Honda and Dubberke, 2009). *C. difficile* has been isolated from many environmental sources such as soil, sand, river bank mud, air and hay (Blawat and Chylinski, 1958, Kim *et al.*, 1981, Al Saif and Brazier, 1996). Thus, the environment is an active reservoir for this pathogen. Its ability to produce spores provides it with an

excellent means of protection to survive the harsh environmental conditions and cleaning (Dawson *et al.*, 2011). Other sources of *C. difficile* include dung and recta of dogs, cats, camels, donkeys, pigs, cows, chickens and horses (Marks and Kather, 2003, Baverud, 2004, Arroyo *et al.*, 2007, Clooten *et al.*, 2008, Zidaric *et al.*, 2008, Alvarez-Perez *et al.*, 2009, Hoffer *et al.*, 2010, Costa *et al.*, 2011). It has also been isolated from vegetables, retail raw meat, the gastrointestinal tract and skin of animals as well as faeces of humans that have never been treated with antibiotics or been hospitalised (Anand and Glatt, 1993, Songer and Anderson, 2006, Songer *et al.*, 2007, Simango and Mwakurudza, 2008, Avbersek *et al.*, 2011). The isolation of *C. difficile* from fresh and cooked animal products such as beef, pork and poultry meat is of zoonotic concern and may have health implications (Clooten *et al.*, 2008, Alvarez-Perez *et al.*, 2009, Weese *et al.*, 2009, Weese, 2010, Weese *et al.*, 2010, Harvey *et al.*, 2011).

1.1.4 Pathogenesis

Clostridium difficile infection is the significant cause of hospital acquired antibiotic-stimulated toxin mediated diarrhoea (Barnett, 2012). Patients who receive care in hospitals, nursing homes and out-patient surgical centres are commonly at risk of CDI due to their low immunity and antibiotic therapies (McMullen *et al.*, 2006). A recent survey revealed that approximately 25 % of CDI cases first show symptoms among patients in hospitals and the remaining 75 % show symptoms among patients from nursing homes or patients who have recently been cared for in GP surgeries or clinics (Voelker, 2012). Voelker (2012) also indicated that 50 % of hospital diagnosed CDI are in patients who were transferred or been recently discharged from other hospitals or care facilities. The pathogen may be transferred to susceptible patients when healthcare workers contaminate their hands or gloves by touching contaminated surfaces, or when patients come into direct contact with contaminated surfaces (Boyce,

2007, Singh, 2009). Therefore, this pathogen has been reported in many parts of the hospital such as emergency rooms and wards (Monsieur *et al.*, 1991, Martirosian *et al.*, 2005b, McMullen *et al.*, 2006, Citak *et al.*, 2011).

CDI is usually considered to be a hospital associated infection. However, this disease may also be acquired by healthy individuals from the environment via the faecal–oral route (Borriello, 1979, 1990, Iizuka *et al.*, 2003, Wilcox *et al.*, 2008) (Figure 1.1). In most community acquired CDI cases, patients have been shown to have received antibiotic therapy or been hospitalised (Wilcox *et al.*, 2008, Khanna *et al.*, 2012, Leffler and Lamont, 2012). In some cases, CDI has been reported even in patients not taking antibiotics (Todd, 2006, Bauer *et al.*, 2008).

CDI starts when the spores are ingested. The normal microbiota in the gut primarily acts as a barrier to colonisation by pathogens but become altered due to the use of broad spectrum antibiotics (Gordon, 2000, Ferreira *et al.*, 2004, Todd, 2006, Elliott *et al.*, 2007). Commonly used antibiotics that can stimulate CDI include aminopenicillin, flucloxacillin, co-amoxiclav, macrolide, trimethropim, clindamycin, cepholosporin and ampicillin (Pierce *et al.*, 1982, Silva *et al.*, 1984, Noren *et al.*, 2002, Merrigan *et al.*, 2003, Wilcox *et al.*, 2008, Buffie *et al.*, 2012). Other antibiotics include moxifloxacin, ciprofloxacin, clarithromycin and cefotaxime (Riley *et al.*, 1991, Braegger and Nadal, 1994, Starr and Impallomeni, 1997, Biller *et al.*, 2005). On reaching the stomach, vegetative cells that might have also been ingested are killed by the acidic gastric juice but the spores are resistant. The spores proceed to the colon where they come in contact with bile salts which stimulate them to germinate and release new vegetative cells. The cells adhere to the gut walls where they grow and multiply (Karjalainen *et al.*, 1994). Disease occurs in the colon due to the anaerobic environment in which this organism lives (Poutanen and Simor, 2004). Colitis results

from a disruption of the normal microbiota of the colon, colonisation with, and the release of *C. difficile* toxins A (ToxA) and toxin B (ToxB) (Figure 1.1) (Borriello *et al.*, 1984, Gerding, 1999, Ho and Ellermeier, 2011).

The *C. difficile* toxins are together responsible for pseudomembranous colitis. ToxA is a potent cytotoxin which causes initial damage to the epithelial lining of the intestine (Sutton *et al.*, 2008, Kreimeyer *et al.*, 2011). The villus tips of the epithelium are destroyed first followed by damage to the brush border membrane (Aktories and Wilkins, 2000). The ToxA opens the tight junction between epithelial cells marked by decreased epithelial resistance, increased bacterial migration and changes in the morphological features of the tight junctions and associated cells (Barth *et al.*, 2004). In addition, there is the production of neutrophils, monocytes, and necrotic enterocytes which appear to play important role in the pathogenesis of CDI (Lyerly *et al.*, 1988, Pothoulakis *et al.*, 1993, Kelly *et al.*, 1994). The gut mucosa becomes denuded and concomittently there is an infiltration of protein-rich exudates such as mucin and fibrin (Poxton *et al.*, 2001).

The second toxin, ToxB is also a potent cytotoxin for epithelial cells. It does not cause damage or fluid response when injected alone in the intestine (Lawrence *et al.*, 1997). It depends on the initial damage caused by ToxA on the surface of intestinal cells (Lyerly *et al.*, 1988, Depitre *et al.*, 1993). This allows ToxB to enter the cells and contribute to the damage during the course of the disease once it gained access to the underlying tissue (Lyras *et al.*, 2009). Proteolytic enzymes and adhesins have been identified as factors that could play a key role in the adherence of these toxins to the gut mucosa (Borriello *et al.*, 1988, Seddon *et al.*, 1990). The epithelial cells become destroyed by apoptosis which leads to complete mucosal damage, colitis,

pseudomembrane formation and diarrhoea (Fiorentini *et al.*, 1998, Voth and Ballard, 2005, Kreimeyer *et al.*, 2011).
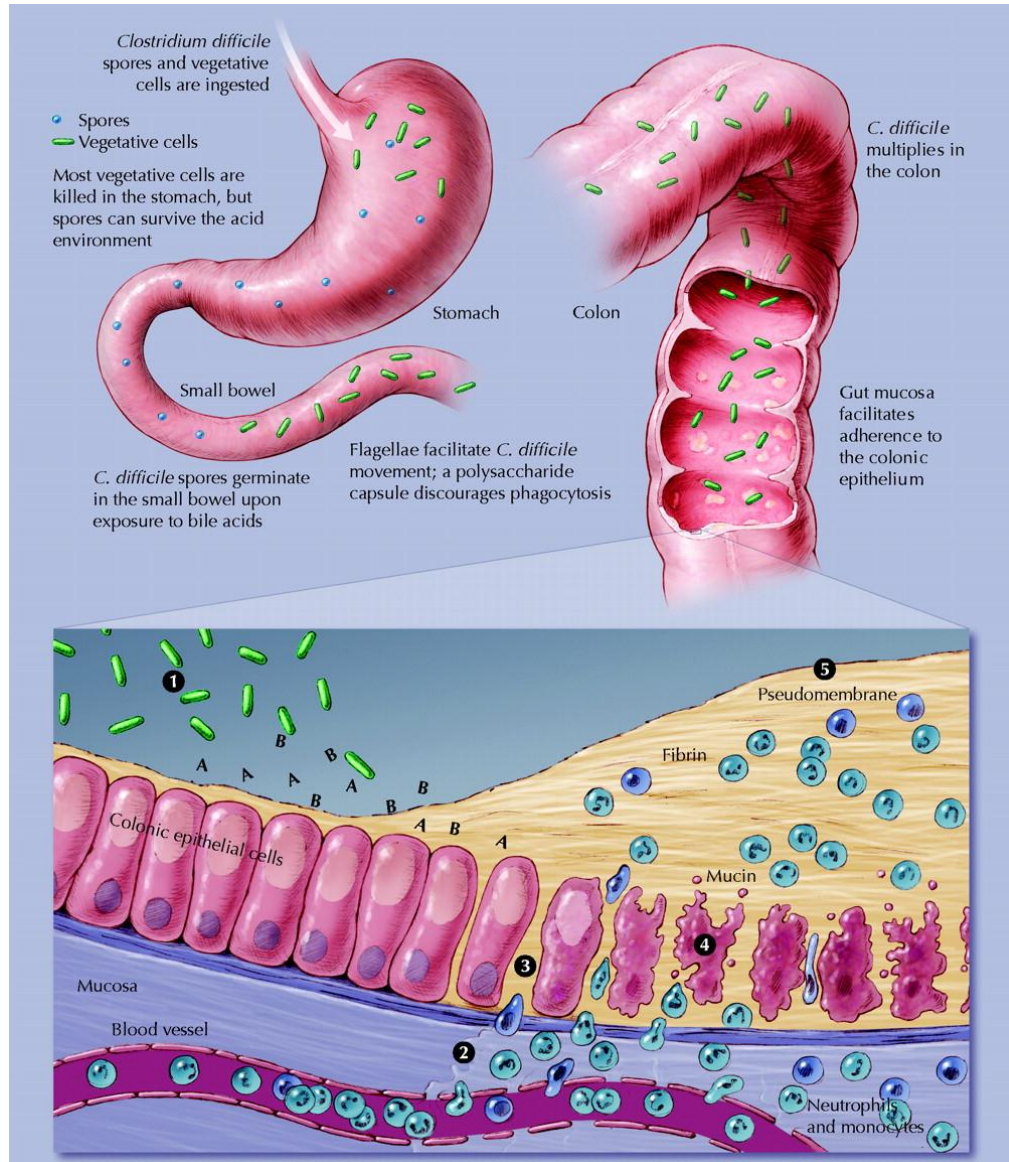
Figure 1.1 Picture showing events after *C. difficile* vegetative cells and spores are ingested leading to pseudomembranous colitis and diarrhea.

http://www.human-healths.com/wp-content/uploads/2011/08/Clostridium-Difficile4.jpeg

When the ingested *C. difficile* spores or vegetative cells reach the stomach, the vegetative cells are killed due to the acidic nature there but the spores persist. In the small intestine, the spores come in contact with the bile salts which stimulate them to germinate releasing new vegetative cells. The vegetative cells proliferate in the large intestine due to the anaerobic condition there and the use of broad-spectrum antibiotics. The *C. difficile* cells release ToxA and ToxB (1) which leads to vascular permeability

and the production of neutrophils, monocytes, and enterocytes (2). The toxins open the tight junction between epithelial cells (3) leading to epithelial apoptosis (4). Local production of hydrolytic enzymes leads to tissue degradation, production of mucin and fibrin leading to pseudomembranous colitis (5) and diarrhoea.

1.1.5 Risk factors

Due to the increasing incidence and severity of CDI, there is the need to reduce the risk factors associated with this disease. The major risk factor is antimicrobial therapy (Hensgens *et al.*, 2012b). Other risk factors include low serum antibody response and other underlying diseases such as lung cancer, leukaemia and renal failure (Bruce *et al.*, 1982, Cunney *et al.*, 1998, Kovithavongs, 1999, Gifford and Kirkland, 2006, Raza *et al.*, 2010, Kosmidis and Chandrasekar, 2012). Other diseases that can predispose patients to CDI include arthritis (Durand and Miller, 2009), pneumonia (Hong and Davis, 1996) and ulcer (Kawaratani *et al.*, 2010, Kariv *et al.*, 2011, Navaneethan *et al.*, 2012). Factors such as old age (Pareja Sierra and Hornillos Calvo, 2007, Goorhuis *et al.*, 2008), organ transplant (Gross *et al.*, 2006, Munoz *et al.*, 2007, Stelzmueller *et al.*, 2007), spinal cord injuries (Dumford *et al.*, 2011), cardiac surgeries (Crabtree *et al.*, 2007) and bone surgeries (Finerty *et al.*, 2006) are associated with CDI. Further exposure to and acquisition of toxigenic *C. difficile* strains and increased hospital stay are additional risks factors associated with CDI (Gerding and Brazier, 1993, Macgowan *et al.*, 1995, Shek *et al.*, 2000, Fletcher and Cinalli, 2007, Hookman and Barkin, 2007). Also, patients with proton pump inhibitors, tube feeding, colectomy, malaria, human immunodeficiency virus (HIV) and ileal infection are at risk of CDI (Golledge and Riley, 1995, Pulvirenti *et al.*, 2002, Dial *et al.*, 2006, Schuler, 2007, Lavallee *et al.*, 2009, O'Keefe, 2010, Turco *et al.*, 2010, Markelov *et al.*, 2011, Tsiouris *et al.*, 2012).

1.1.6 Symptoms of CDI

The Department of Health (DH) has defined *C. difficile* infection as one episode of diarrhoea, either as stool loose enough to take the shape of a container used to sample it or as Bristol Stool Chart types 5-7. The diarrhoea should not be attributable to any other cause, including medicine, and occurs at the same time as a positive toxin assay (with or without positive culture and/or endoscopic evidence of PMC) (Department of Health and HPA, 2008). However, *C difficile* infection can result in either symptomatic or asymptomatic conditions (Feingold *et al.*, 1979, Donta and Myers, 1982). For symptomatic carriers, the incubation period ranges from 1 to 6 days after antibiotic exposure (Sunenshine and McDonald, 2006). Other symptoms associated with CDI include moderate to severe lower abdominal pain and systemic symptoms such as fever, anorexia, nausea and malaise (Gerding and Brazier, 1993). In few instances, leukocytosis and faecal occult blood can occur (Bartlett, 2000, Malnick and Zimhony, 2002, Reddymasu *et al.*, 2006). An endoscopy of the colon shows white to yellow plaques of varying diameters due to accumulation of the bacterial debris, necrotic colonic walls and fibrinous exudates containing dead leucocytes, epithelial cells and mucin (Figure 1.2) (Kelly and LaMont, 1998, Sunenshine and McDonald, 2006, Kelly, 2007, Kelly and LaMont, 2008).
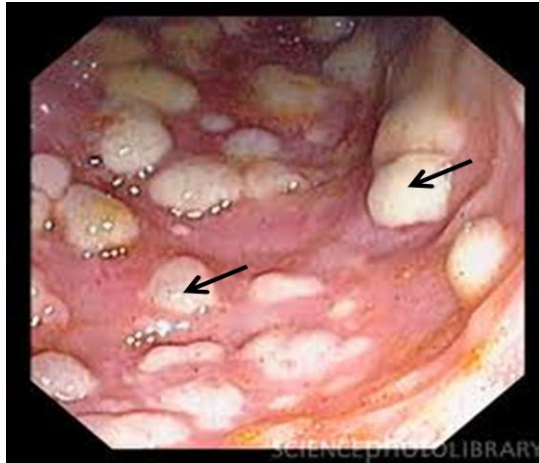
Figure 1.2 Pseudomembranous colitis.

http://www.sciencephoto.com/image/252788/530wm/M1300688-Colitis-SPL.jpg.

The picture above shows an endoscopy of an inflamed large intestine with pseudomenbronous colitis, a characteristic manifestation of full-blown *C. difficile* colitis. Classic pseudomembranes are visible as raised white-yellow plaques (indicated by black arrows) ranging from 2-10 mm in diameter and scattered over the colorectal mucosa.

Symptoms of CDI may worsen and become life threatening with dilation of the colon leading to toxic megacolon. In addition to the symptoms mentioned above, toxic megacolon can include sepsis, paralysis of the ileum, loss of colonic muscular tone, colonic perforations and peritonitis (Malnick and Zimhony, 2002, Barbut *et al.*, 2007, Berman *et al.*, 2008, Autenrieth and Baumgart, 2011). In the toxic mega colon, fatality can increase to 32-50 % (McFarland, 2005, Autenrieth and Baumgart, 2011, Griniatsos *et al.*, 2011).

Beside symptomatic carriers, other CDI patients can be asymptomatic. The asymptomatic carriers include infants, children as well as healthy adults (Shim *et al.*, 1998, Banaszkiewicz *et al.*, 2011). The majority of hospitalised patients infected by *C. difficile* are asymptomatic carriers, who serve as silent reservoir for continued *C. difficile* contamination of the hospital environment. The carriers seldom develop symptomatic disease that is the carrier state is stable until the colonic flora is disrupted

as described in section 1.1.4. Previously, CDI was thought to be the disease of the elderly with infants being colonised but remained asymptomatic (Cashore *et al.*, 1981, Donta and Myers, 1982, Bolton *et al.*, 1984). Two reasons have been proposed to explain the asymptomatic CDI in infants. The reasons include the lack of toxin receptors, and protection by colostrum in breast milk which contains some anti-toxigenic activities against the *C. difficile* toxins (Kim *et al.*, 1984, Lyerly *et al.*, 1988). However, the epidemiology of the disease is changing quite rapidly. A recent study has shown that children who formed majority of the low risk groups are becoming infected with CDI (Pituch, 2009). Reasons for the change in CDI in paediatric patients include disruption of the normal microflora of the gastrointestinal tract, age, immune status, diet, underlying health conditions, concurrent infections, and cancer (Pituch, 2009).

1.1.7 Treatment of CDI

Presently, the traditional method for the treatment of CDI relies on vancomycin and metronidazole but resistance to both antibiotics has been reported (Aslam *et al.*, 2005, Pepin, 2008). In 2007 and 2008, metronidazole remains the antibiotic of choice for treating patients with initial CDI considering its low cost (Pepin, 2008). Vancomycin was reserved for more severe cases or in pregnant women due to the risks of congenital abnormalities, preterm delivery and low birth weight associated with metronidazole (Sørensen *et al.*, 1999, McFarland, 2005). However, in 2009 and 2010, there appeared to be a shift to vancomycin treatment for CDI due to the marked reduced susceptibility of isolates to metronidazole (Freeman *et al.*, 2007, McFarland, 2009b, HPA, 2010). Although McFarland (2005) reported that there were no differences in the efficacies of the two antibiotics, a decreased susceptibility of *C. difficile* strains such as ribotypes 027, 106 and 017 to metronidazole has been reported with clusters of 027 strains having varied susceptibility (McFarland, 2005, 2009b, HPA, 2010).

Due to the complications associated with the two antibiotics described above, other alternative treatments using non-antibiotic approaches for this disease were reported. One of such treatments includes the use of probiotics in conjunction with antibiotics. The probiotics help in restoring the gut microbiota and thereby increasing colonisation resistance in the gut. The restored organisms compete with *C. difficile* for essential nutrients, mucosal adherence sites and space in the gut thereby preventing CDI (Isakow *et al.*, 2007, Bauer and van Dissel, 2009, Johnson, 2009). Another alternative is the use of toxin binding resins and polymers such as colestipol and tolevamer (Mogg *et al.*, 1982, Louie *et al.*, 2006, Barker *et al.*, 2007, Bauer and van Dissel, 2009). This treatment strategy uses the concept of the fact that *C. difficile* does not cause disease unless its toxins bind to the mucosal lining and cause damage. The resins and polymers bind to the toxins thus preventing infection. Trial of the use of colestipol was criticised as only half of the participants had *C. difficile* toxins detected in their stool. Hence, the participants might not have been infected in the first place with the disease (Bauer and van Dissel, 2009). The tolevamer however was more successful and was found be as effective as vancomycin and well tolerated by the participants. However, it was found to be associated with increased risk of hypokalemia (low potassium in the blood) (Louie *et al.*, 2006). Immunotherapy, where neutralising antibodies to *C. difficile* toxins are administered to patients lacking adequate amount of antitoxin antibodies, has been shown to help improve clinical recovery of CDI (McFarland, 2005, McPherson *et al.*, 2006, Hassoun and Ibrahim, 2007, Binion, 2011). Another method of treating CDI is through faecotherapy which involves transplanting a healthy donor faecal matter into an infected patient via the colonoscope or nasal tube (Aas *et al.*, 2003, Bakken, 2009, Bauer *et al.*, 2009, Hundal *et al.*, 2011). The faecal transplant restores gut microbiota and reduces *C. difficile* colonisation and symptoms thus, preventing recurrence

(Bowden *et al.*, 1981, Persky and Brandt, 2000, van Nood *et al.*, 2009). Other promising methods of treating CDI include the use of phage and phage derived products or brewer's yeast (Seal *et al.*, 1989, Schellenberg *et al.*, 1994, Ramesh *et al.*, 1999, Mayer *et al.*, 2008, Meader *et al.*, 2010, Patrick, 2011). In addition, patient's isolation, restrictive antibiotic administering, accurate and rapid diagnosis, appropriate treatments and implementation of enteric precautions for symptomatic patients will help prevent CDI. Environmental disinfection and simple good hygiene practice such as washing of hands before and after using the toilets or coming in contact with patients are also very effective in reducing CDI (Skoutelis *et al.*, 1994, Gerding, 1999, Wilcox and Fawley, 2000, Barbut and Petit, 2001, Gopal Rao *et al.*, 2002, Sule *et al.*, 2006, Dumford *et al.*, 2009).

1.1.8 Epidemiology of CDI

CDI is a global challenge and its prevalence and epidemiology is changing very rapidly (Chaudhry *et al.*, 2008, Byun *et al.*, 2009, Dale and Gerding, 2010). Currently, 48 % of CDI reported cases and 11 % death cases in Europe are attributed to *C. difficile* PCR ribotype 027 (Wiegand *et al.*, 2012). This ribotype was first reported in 2005 in the Stock Mandeville Hospital and later in Netherlands (Smith, 2005, Kuijper *et al.*, 2006b). It is common in Europe and North America (Warny *et al.*, 2005, Kuijper *et al.*, 2006a, Kuijper *et al.*, 2007, Kuijper *et al.*, 2008). It has been reported in parts of the United Kingdom such as Scotland, England and Wales (Djuretic *et al.*, 1999, Green *et al.*, 2007, Mutlu *et al.*, 2007, Reddy *et al.*, 2010). This strain was also reported in the USA (McDonald *et al.*, 2006, Kim *et al.*, 2008), Quebec, Canada (Pepin *et al.*, 2004, Loo *et al.*, 2005, Mulvey *et al.*, 2010), Italy (Spigaglia and Mastrantonio, 2004, Mellace *et al.*, 2012), Austria (Indra *et al.*, 2006, Indra *et al.*, 2009), France (Coignard *et al.*, 2006, Coignard *et al.*, 2007, Eckert and Barbut, 2010), Australia (Richards *et al.*, 2011),

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Finland (Lyytikainen *et al.*, 2007), Republic of Ireland (Drudy *et al.*, 2007, Long *et al.*, 2007, Fitzpatrick *et al.*, 2008), Germany (Reichardt *et al.*, 2007, Zaiss *et al.*, 2007, Kola, 2010), Singapore (Lim *et al.*, 2011), Israel (Bishara *et al.*, 2011), Korea (Kim *et al.*, 2007, Kim *et al.*, 2011), Japan (Iwashima *et al.*, 2010), China (Liu *et al.*, 1997), Poland (Martirosian *et al.*, 1993, Martirosian *et al.*, 2005a), Sweden (Noren *et al.*, 2002) and Hungary (Terhes *et al.*, 2009).

In the UK, CDI has become a national health problem. Across England, Wales and Northern Ireland there was an increase in prevalence of CDI in 2007 (57,255 reported cases of which 20 % were in the younger age groups (<2 years) that were previously not at risk (HPA, 2010) (Figure 1.3). The rise in CDI cases in 2007 was attributed to insufficient awareness and problems in the implementation of the 1994 guidelines on the prevention and management of *C. difficile.* Other reasons were due to changes in health care practices including an increase testing and patient risk profile particularly in relation to age. More importantly, the appearance of the more virulent strain, the ribotype 027 also known as the North America pulse field type 1 (NAP1) or B1 by restriction endonuclease analysis is involved in a more severe cause, antibiotic resistance, higher mortality and more increased risk of relapse (Warny *et al.*, 2005, Kuijper *et al.*, 2007, van der Kooi *et al.*, 2008).

The set target by the HPA was to reduce CDI by 30 % in 2008. This set target was achieved with a tremendous decrease by 38 % of reported cases in England, 0.8 % in Wales and an increase of 20.8 % in Northern Ireland. The marked decrease in England was attributed to restrictive antibiotic prescribing where only narrow-spectrum agents for empirical treatment were allowed. Also the avoidance of clindamycin and second- and third-generation cephalosporins especially in the elderly, the minimisation of the use of fluoroquinolones, carbapenems and prolonged course of aminopenicilins

have helped to reduce CDI during this period (HPA, 2008). Other reasons for the marked decrease of CDI in 2008 were associated with patient isolation and changes in cleaning and disinfection of the hospital environment. Alcohol based cleaning agents were substituted for chlorine and bleach based agents. Hand washing which mechanically removes spores and vegetative cells was encouraged instead of the use of alcohol hand-rub that encourages sporulation in *C. difficile.*

According to the HPA, CDI cases in 2009-2011 in NHS acute Trusts have declined dramatically as shown in Figure 1.3. Nevertheless, the total number of cases of patients from 2 years and above (4,828), 60 years and above (3,782) and trust apportioned (2,353) in January-March 2011 still remain considerably high (Figure 1.3). The HPA also indicated that there is a changing prevalence of ribotypes but 027 still remains most dominant ribotype and contributes to about 48 % of the reported cased in 2011. Due to the hypevirulent nature of ribotype 027, there is an urgent need to identify factors that contribute to the success of this strain and the epidemic potential of the new ones. Some of the virulent factors of *C. difficile* are discussed below.
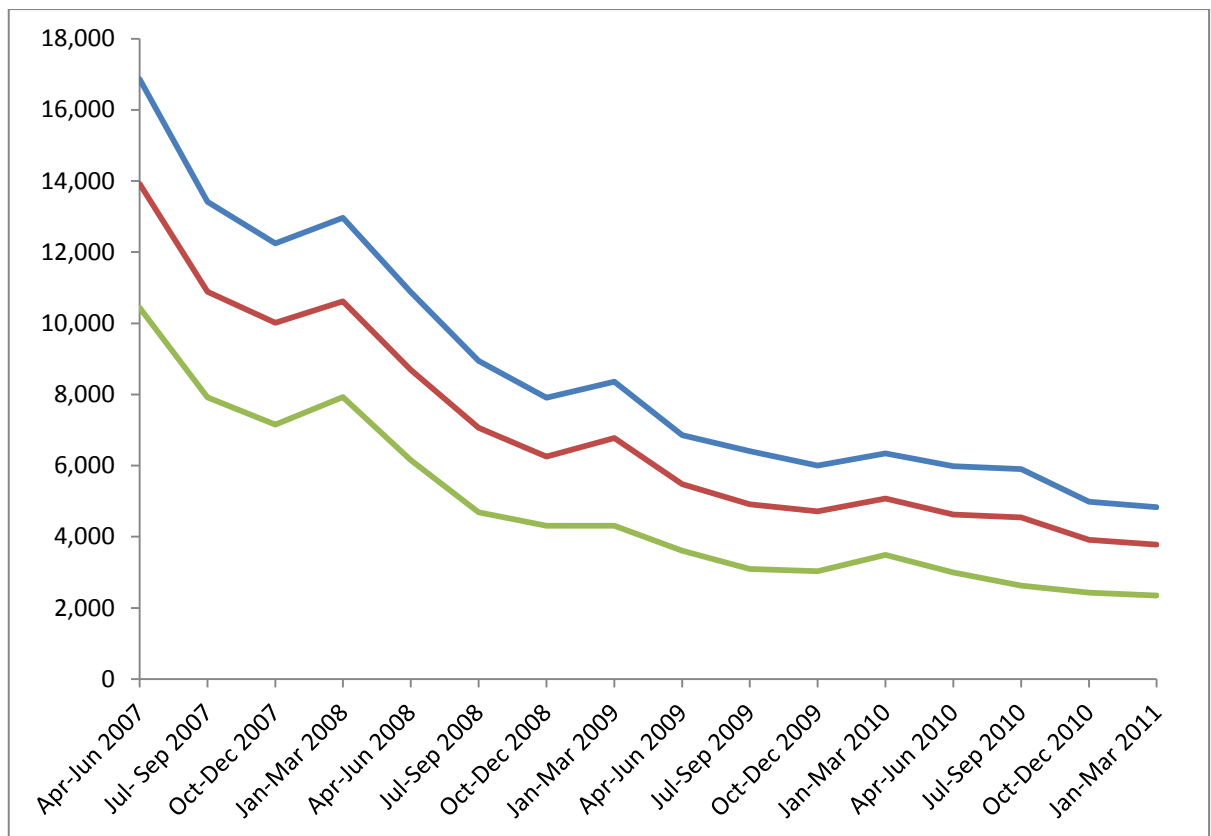
Figure 1.3 HPA quarterly report of CDI from 2007 to 2011

The summary of HPA quarterly report of CDI in England from 2007-2011 is shown in the graph above. Total CDI reports in children age ≥2 years ——— Total CDI reports in patients ≥65 years ——— Trust apportioned CDI reported cases in patients ≥2 years ———

1.1.9   Virulence factors of *C.difficile*

1.1.9.1   The Toxin A and B

The pathogenicity of *C. difficile* is linked to a 19.6 kb genetic locus found mainly in the chromosome of pathogenic strains (Braun *et al.*, 1996, Cohen *et al.*, 2000) (Figure 1.4). This pathogenic locus known as PaLoc is associated with genes responsible for toxin production (Hammond and Johnson, 1995, Hammond *et al.*, 1997, Dingle *et al.*, 2011). The two toxins TcdA (ToxA) and TcdB (ToxB), encoded by the *tcdA* and *tcdB* genes respectively, are the key virulence factors associated with this organism, and each exists as a single open reading frame of the pathogenic locus

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(Lyerly *et al.*, 1988, Voth and Ballard, 2005). Three other additional genes in the PaLoc (*tcdC*, *tcdD* and *tcdE*) are considered to play important roles in the control of the virulence of *C. difficile* (Lyerly *et al.*, 1982, Voth and Ballard, 2005).

The open reading frames of *tcdA* and *tcdB* encode 8,133 and 7,098 nucleotides respectively (Figure 1.4) (Voth and Ballard, 2005). ToxA (308 kDa) and ToxB (270 kDa) are glycosyltransferases and among the largest bacterial toxins reported (Farrell and LaMont, 2000). Other widely studied clostridial toxins include *Clostridium sordellii* lethal toxin (TcsL) and hemorrhagic toxin (TcsH) (Chang *et al.*, 1978, Voth *et al.*, 2004), *C. perfringens* enterotoxin (CPE) (Vaishnavi and Kaur, 2008) and *Clostridium novyi* alpha toxin (Tcnα) (Spyres *et al.*, 2003).

In *C. difficile,* the expression of ToxA and ToxB is highest in the late log phase or stationary phase in response to environmental conditions (Lyerly *et al.*, 1982, Voth and Ballard, 2005). Thus, the glycosylating activity of ToxA and ToxB controls numerous physiological activities of the cell thereby directly contributing to pathogenicity of the disease (Voth and Ballard, 2005). Primarily, it was reported that ToxA not ToxB was responsible for PMC because ToxB was unable to cause the disease until ToxA was present (Lyerly *et al.*, 1988, Depitre *et al.*, 1993, Lyras *et al.*, 2009). In a study on hamsters, the animals had to be immunised against both ToxA and ToxB before they became protected against the disease (Fernie *et al.*, 1983). Therefore, both toxins have to be present to cause disease. In another report, ToxA^{-} ToxB^{+} strains were found to be present in clinical samples and have been responsible for fatal cases of CDI (Al-Barrak *et al.*, 1999). This indicates that ToxB also contributes to the disease or can substitute for the ToxA deficiency and consequently the development of the disease in ToxA-deficient strains (Spyres *et al.*, 2003, Elliott *et al.*, 2011).

The *tcdC* is found downstream of *tcdA* on the PaLoc (Figure 1.4) and is transcribed in the opposite direction from *tcdA* and *tcdB*. *TcdC* is expressed in early exponential phase but declines as growth reaches stationary phase (Hundsberger *et al.*, 1997). The decrease in TcdC expression in this phase corresponds with the increase in ToxA and ToxB signifying that *tcdC* negatively regulates *tcdA* and *tcdB* (Spigaglia and Mastrantonio, 2002, Dupuy *et al.*, 2008).

The *tcdD* is located upstream of *tcdB* and also affects the expression of *tcdA* and *tcdB* (Figure 1.4). TcdD is similar to DNA binding proteins and has been shown to enhance expression of promoter reporter fusions containing the promoter binding regions of *tcdA* and *tcdB* (Braun *et al.*, 1996, Hundsberger *et al.*, 1997, Dupuy *et al.*, 2008). TcdD has been shown to resemble TetR and BotR which are positive toxin regulators in *C. tetanus* and *C. botulinum* respectively (Dupuy *et al.*, 2005, Raffestin *et al.*, 2005). The expression of TcdD is repressed by glucose and expression of this toxin greatly increases as stationary phase progresses (Braun *et al.*, 1996, Hundsberger *et al.*, 1997). This corresponds with an increase in ToxA and ToxB. Thus *tcdD* is a positive regulator of toxin expression in *C. difficile*.

tcdE is another gene found in the pathogenic locus and is situated between *tcdA* and *tcdB* (Figure 1.4) (Voth and Ballard, 2005). It was shown to be homologous with holin protein and as such found to facilitate the release of ToxA and ToxB through changing the permeability of the cell wall of *C. difficile* (Tan *et al.*, 2001). The phage holin-like function of *tcdE* was reported to be expressed in *Escherichia coli* (Wang *et al.*, 2000, Wang *et al.*, 2003).
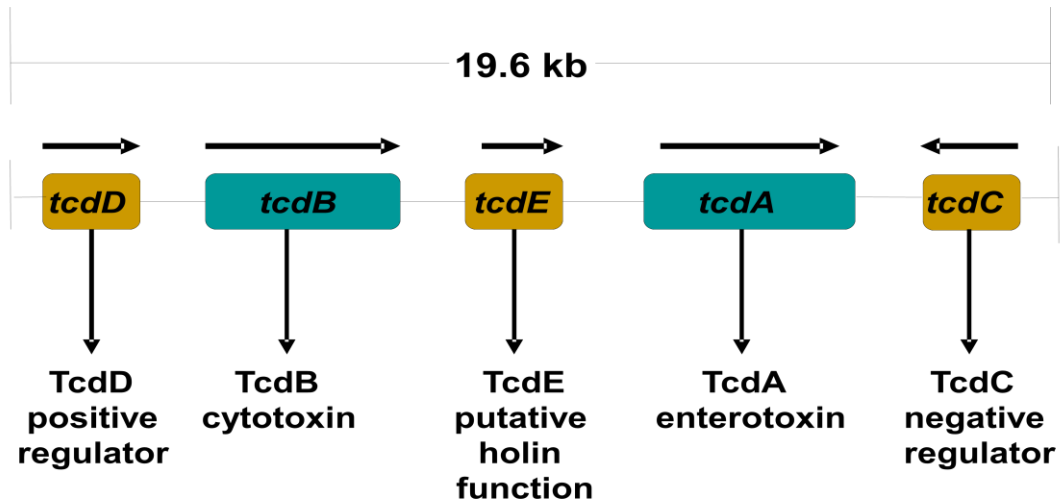
Figure 1.4 The Pathogenic locus (PaLoc) of *Clostridium difficile* (Voth and Ballard 2005).

Above is the schematic representation of the 19.6 kb *C. difficile* PaLoc showing *tcdA* and *tcdB* genes encoding the two major toxins (TcdA and TcdB respectively), the negative and positive regulatory genes (*tcdC* and *tcdD* respectively) and the holin-like gene (*tcdE*).

1.1.9.2 The *C. difficile* binary toxin

In addition to ToxA and ToxB, it was reported that some strains of *C. difficile* produce other toxins carried by genes outside the PaLoc (Perelle *et al.*, 1997, Carter *et al.*, 2007). Carter *et al.* (2007) reported that the *C. difficile* binary toxin (CDT) is located in the *C. difficile* binary toxin locus (CdtLoc). The study revealed that CdtLoc is lacking in the non-toxigenic strains (such as CD37) (Figure 1.5A) but is present and approximately 4.2 kb in binary toxin-negative ones (such as CD630) (Figure 1.5B) and 6.2 kb in the chromosome of binary toxin-positive strains (such as QCD-32g58) (Figure 1.5A). The CdtLoc encodes *cdtA* and *cdtB* (Figure 1.5C), which are toxin genes responsible for the production of the CDT subunits CDTa and CDTb. The two subunits were shown to form the enzymatic component and binding component of CDT, respectively (Barth *et al.*, 2004). A third gene in the CdtLoc is the *cdtR* located upstream of *cdtA* (Figure 1.5C) and encodes the regulatory gene responsible for the

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optimal regulation of the binary toxin, a function similar to *tcdD* in the PaLoc (Perelle *et al.*, 1997). The report of Carter *et al.* (2007) further revealed that the functional *cdtR* resulted in maximum expression of the binary toxin whereas a deletion in it resulted in the reduction of binary toxin expression. The binary toxin negative strain was shown to carry the pseudogenes *cdtAB* in place of the *cdtA* and *cdtB* at the 3' end of the CdtLoc (Figure 1.5B). It was reported that *cdtAB*, *cdtA* and *cdtB* were lacking in the non-toxigenic strains but in their place was a gene of 68 bp with unknown function (Figure 1.5A) (von Eichel-Streiber *et al.*, 1990, 1992, Carter *et al.*, 2007). The possession of a binary toxin gene in addition to ToxA and ToxB can result in an increase in toxin production leading to high CDI severity as previously reported (Bacci *et al.*, 2011).

A

AAAAAATAAA**ACATTACTTAATTTAAGTTTATTATTCTTATAATAAATTAGATACATAAAAATAAATTCCATAATTAT**TGTATCATAT
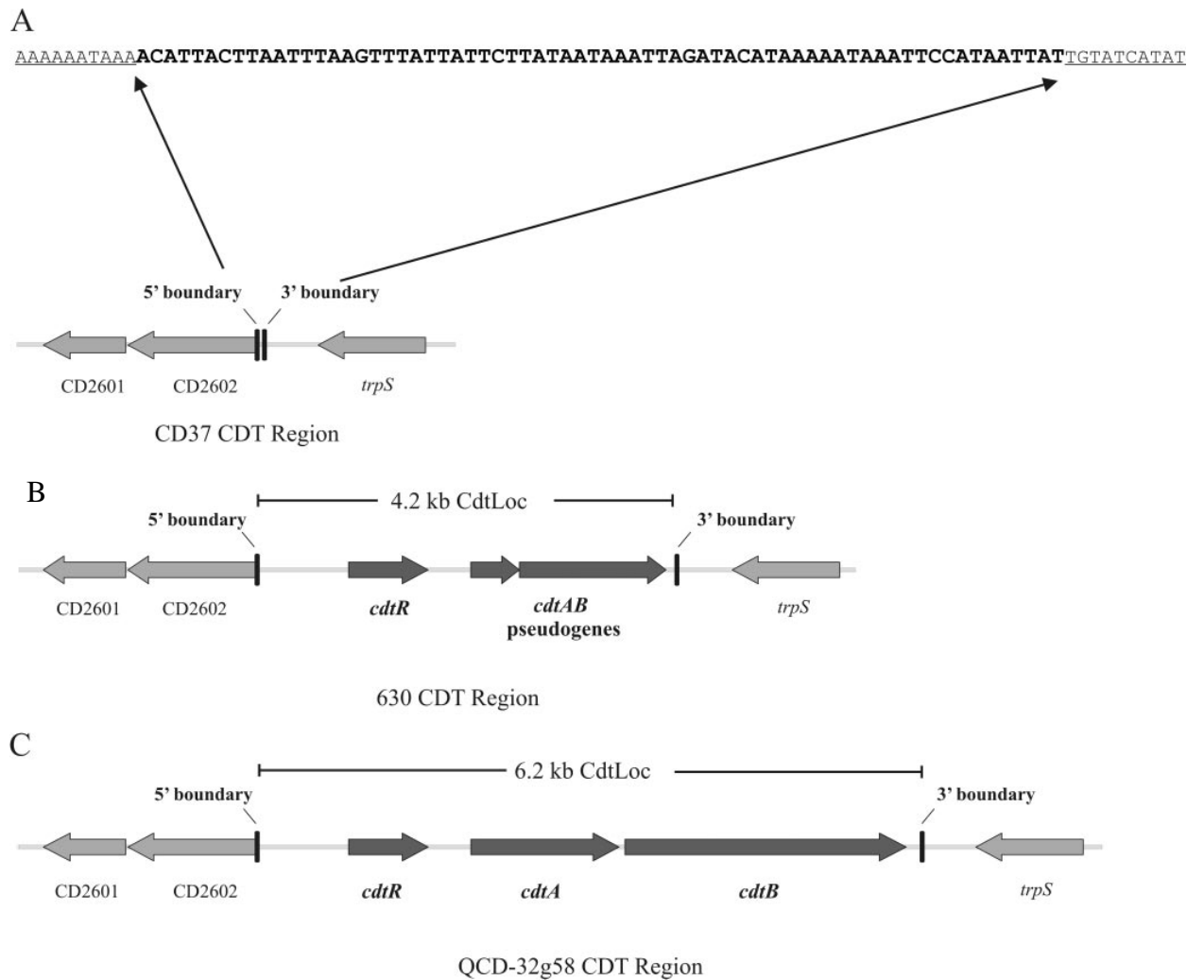
Figure 1.5 A schematic representation of *Clostridium difficile* binary toxin (CDT) genes and flanking regions (Carter *et al.*, 2007).

The three variants (A, B and C) of the binary toxin (CDT) are shown in the diagram above. The regions of the representative non-toxigenic (A), binary toxin negative (B) and binary toxin positive (C) strains are shown. The CdtLoc comprised of 6.2 kb in binary toxin positive strains (e.g. QCD-32g58), 4.2 kb in binary toxin negative strains (e.g. CD630) and lacking in non-toxigenic strains (e.g. CD37). For each variant of the CDT region, the positions of the 5′ and 3′ conserved boundaries are shown, and the size of the entire CdtLoc is indicated. The unique 68 bp sequence that is present in CD37 and other non-toxigenic strains in place of the CdtLoc is shown in bold.

1.1.10 Other virulence factors

1.1.10.1 Adhesin

Adhesion to host tissue is important for full expression of virulence in many pathogens. *C. difficile* have been shown to have the ability to adhere to gut surfaces. The first report to show this was in 1979 (Borriello, 1979). In this report, microscopic examination of washed biopsy specimen of a patient with PMC showed the presence of *C. difficile* cells. In a similar experiment on a hamster model, it was shown that a highly virulent toxigenic strain adhered better than a poorly virulent strain, and both strains adhered better than an avirulent non-toxigenic strain (Borriello *et al.*, 1988). Adherence was shown to be more pronounced in the terminal ileum and caecum where the disease is more prominent. Borriello *et al.* (1988) also showed that the co-administration of non-toxigenic strains with *C. difficile* toxin A raised the adhesin of the former to the same level as that of a toxigenic one. This implies that adhesion is facilitated by toxin A mediated damage or that toxin A is directly involved in binding *C. difficile* to the gut as discussed in section 1.1.4. Many factors can contribute to the adhesion of *C. difficile* to the gut. These include:

a. **Fimbriae:** Fimbriae are proteinaceous appendages found in bacteria. *C. difficile* are motile and have fimbriae which are polar, about 4-9 nm in diameter and 6 µm in length (Borriello *et al.*, 1988). The flagella, composed of the flagellin FliC and the flagellar cap protein FliD, have been reported to be involved in intestinal mucus attachment (Tasteyre *et al.*, 2000). The *C. difficile* vegetative cells bind to the human intestinal epithelial cells (Caco-2 and HT-29MTXC) and specific components of the extracellular matrix such as fibrinogen, laminin, fibronectin, collagen I, III and IV. (Eveillard *et al.*, 1993, Karjalainen *et al.*, 1994, Cerquetti *et al.*, 2002). Two heat-stimulated 27 kDa and 40 kDa proteins

were identified from *C. difficile* cells and they appeared to be involved in adhesion to the epithelial cells (Eveillard *et al.*, 1993, Karjalainen *et al.*, 1994). When cloned and expressed, the proteins were able to promote adhesion in *E. coli*. The fimbriae have also been shown to be affected by environmental factors such as temperature and pH (Karjalainen *et al.*, 1994). Recently, a similar report showed that spores of *C. difficile* were able to adhere to the human intestinal epithelial cells through the help of two proteins of approximately 40 kDa molecular weight (Paredes-Sabja and Sarker, 2012).

b. **Surface layer proteins:** The *C. difficile* vegetative cell expresses two major cell surface proteins known as the S- layer proteins. These proteins may act as adhesins and facilitate interaction between the *C. difficile* cells and gut cells (Calabi *et al.*, 2002). The S-layer protein contains two main layers with different molecular weights (Kawata and Masuda, 1984, Masuda *et al.*, 1989). The two sub-units comprise of the high molecular (48-56 kDa) and the low molecular weight (37-45 kDa) proteins (McCoubrey and Poxton, 2001, Poxton *et al.*, 2001, Fagan *et al.*, 2009). The Cwp66 protein of 66 kDa molecular weight was the first S-layer protein to be identified in *C. difficile* and functions as an adhesin (Waligora *et al.*, 2001). The Cwp66 contains two domains, each carrying three imperfect repeats and one presenting homologies to the autolysin CwlB of *Bacillus subtilis* (Waligora *et al.*, 2001). Therefore, S-layer proteins of *C. difficile* are highly variable and may contribute to the virulence of the organism by promoting adherence and colonisation or inducing inflammatory and immunological host response (McCoubrey and Poxton, 2001, Poxton *et al.*, 2001, Fagan *et al.*, 2011, Ryan *et al.*, 2011). S-layers have also been reported in many bacterial pathogens including *Wolinella recta* and *Bacillus cereus* and it is

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assumed that they play a role in pathogenicity and virulence (Borinski and Holt, 1990, Kotiranta *et al.*, 1998, Sleytr and Beveridge, 1999).

c. **Physicochemical properties:** The physicochemical properties of microorganisms can contribute to its ability to adhere to surfaces. *C. difficile* cells are hydrophilic and carry a net positive charge which is evenly distributed on the cell wall (Krishna *et al.*, 1996). Charged interactions with the negatively charged human gut can lead to colonisation (Waligora *et al.*, 1999).

1.1.10.2 Enhanced Sporulation and germination

As mentioned in section 1.1.1, *C. difficile* is able to form endospores which are stable in the environment and in the gut (Akerlund *et al.*, 2008). The spores are resistant to harsh environmental conditions, heating, desiccation and disinfection (Dawson *et al.*, 2011). Specific ribotypes (and strains within a ribotype) of *C. difficile* produce large amount of spores which play a significant role in their survival and spread in the hospital environment (Wilcox and Fawley, 2000, Akerlund *et al.*, 2008). Some Clostridial toxins like those of *C. botulinum* and *C. perfringens* are associated with spore production (Yamakawa *et al.*, 1983, Zimmer *et al.*, 2002). Whether sporulation induces toxin increase in *C. difficile* has not yet been proven although the non-sporulating mutant of *C. difficile* has been reported to produce the same amount of toxin as the spore producing ones (Bennett *et al.*, 1989). Reports on sporulation rates among *C. difficile* strains are discussed further in section 5 of this thesis.

1.1.10.3 Hydrolytic enzymes

Hydrolytic enzymes break down protein, carbohydrate, and fat molecules into their simplest units. The hydrolysis of polymers by hydrolytic enzymes results in free monomers. There has been sporadic work on the production of hydrolytic enzymes by *C. difficile*. One report examined 21 isolates of *C. difficile* and found that all were

positive for hyaluronidase activity, though the amounts produced were variable (Hafiz and Oakley, 1976). Steffen and Hentges (1981) examined one isolate of *C. difficile* in a study of hydrolytic enzyme production by anaerobes from human infections. The isolate was positive for hyaluronidase, chondroitin-4-sulphatase, gelatinase and collagenase, but negative for heparinase, fibrinolysin, lecithinase and lipase (Steffen and Hentges, 1981). The most detailed study of hydrolytic enzymes that may be involved in breakdown of connective tissue was undertaken by Seddon *et al.* (1990). This report showed that most of the strains of *C. difficile* examined had hyaluronidase, chondroitin-4-sulphatase and heparinase activity, though the heparinase activity was generally weak (Seddon *et al.*, 1990). Collagenase activity was also observed in *C. difficile*, but was generally weak and more restricted to highly virulent strains. It is possible that some of these tissue degradative enzymes contribute to the observed pathology and help to compromise further gut integrity and subsequent fluid accumulation during infection (Seddon *et al.*, 1990).

1.1.10.4 Bacteriophages carriage

Another important factor that can influence toxin production is the acquisition of extra chromosomal DNA elements such as plasmids, transposons or bacteriophages (Sebaihia *et al.*, 2006, Henn *et al.*, 2010). Temperate bacteriophages have been shown to play several key roles in the evolution of pathogenic bacteria by either supplementing or modifying toxin production (Brussow *et al.*, 2004). Studies have shown that phage infection of toxigenic *C. difficile* strains can cause an increase in toxin production (Goh *et al.*, 2005a, Govind *et al.*, 2009, Sekulovic *et al.*, 2011). In addition, research at the University Hospitals of Leicester (UHL) showed that there is an association between temperate bacteriophage carriage and clinical outcome when 129 patients isolates were examined (Tromans *et al.*, 2010). Similarly, the examination of 91 clinical strains of *C.*

difficile 027 showed that there is a correlation between phage carriage and diversity that exist in this ribotype (Nale *et al.*, 2012). Report on other Clostridial species such as *Clostridium perfringens* reveal that there may be a link between phage carriage and sporulation (Zimmer *et al.*, 2002). Bacteriophages are discussed in full in sections 1.2 and 3 of this thesis.

1.1.11 Ribotypes of *C. difficile*

There are more than 430 ribotypes of *C. difficile* and these are widely distributed (Warren Fawley pers comm). Ribotypes are restriction fragment length polymorphism (RFLP) analysis of rRNA genes that are used to differentiate between strains of *C. difficile* (Bidet *et al.*, 1999, Brown *et al.*, 2007). Ribotyping uses polymerase chain reaction to amplify the *C. difficile* intergenic spacer region of 16S - 23S operon of the rRNA (rrn) (Song *et al.*, 2002, Bouchet *et al.*, 2008). Several copies of this operon are produced and the differences in sizes of the spacer region also vary. This results in the production of different banding patterns of varying weights which are resolved on an agarose gel and this has been used to differentiate between *C. difficile* ribotypes (O'Neill *et al.*, 1996, Indra *et al.*, 2010, Hell *et al.*, 2011). Within the 430 ribotypes found in Europe, only about 20 % are circulating and are clinically relevant. Some of the clinically relevant ribotypes include 014/020, 001, 078, 018, 106, 027, 002, 012, 017, 015, 126, 023, 046, 003, 011, 053 and 056 (Coignard *et al.*, 2006, Kuijper *et al.*, 2007, Martin *et al.*, 2008, Miyajima *et al.*, 2011). Ribotypes 018, 056 and 027 have been shown to be associated with complicated disease outcome (Tromans *et al.*, 2010, Baines *et al.*, 2011, Bauer *et al.*, 2011, Dingle *et al.*, 2011). Most importantly, ribotype 027 has been reported to exist in subclades with varying disease severities which complicates epidemiological studies (Tachon *et al.*, 2006, Fawley *et al.*, 2008). This ribotype has been shown to be the most commonly acquired strain and emerges

sporadically under antibiotic pressure during hospitalisation (van der Kooi *et al.*, 2008, Buffet-Bataillon *et al.*, 2012).

1.1.11.1 The *C. difficile* ribotype 027

C. difficile 027 was first reported in 2005 in England and shortly thereafter in Netherlands (Smith, 2005, Kuijper *et al.*, 2006b). This epidemic hypervirulent strain of *C. difficile* is characterised as toxinotype III, North American pulsed-field gel electrophoresis type 1, restriction endonuclease analysis type B1 and PCR ribotype 027 (NAP1/027) (Coignard *et al.*, 2006, Blanckaert *et al.*, 2008). Ribotype 027 has been shown to carry the binary toxin gene in addition to the 18 bp and 1 bp deletions in the negative regulatory gene *tcdC*. These deletions result in frame-shift mutations in the PaLoc leading to the premature termination of the transcription of the negative regulatory gene and consequently leading to higher production of toxins (Loo *et al.*, 2005, Bacci *et al.*, 2011). In addition, this ribotype is fluoroquinolone resistant and readily forms endospores (Akerlund *et al.*, 2008, Kuijper *et al.*, 2008, Merrigan *et al.*, 2010, Vohra and Poxton, 2011). It is also associated with more severe disease and increased therapeutic failures which results in higher mortality, colectomy rates and increased risk of relapse (Wilcox and Spencer, 1992, Loo *et al.*, 2005, McDonald *et al.*, 2005, Sun *et al.*, 2011). The widespread nature of this ribotype in North America and Europe has significantly altered the epidemiology of CDI with 027 strains becoming dominant (Barbut *et al.*, 2002, McConnell, 2007, Marsden *et al.*, 2010).

There are increasing questions as to whether ribotype 027 strains cause more severe disease than other ribotype strains. A study compared the clinical outcome of patients infected with *C. difficile* 027 and those infected with non-027 strains (Morgan *et al.*, 2008, Karlowsky *et al.*, 2012). Morgan *et al.* (2008) reported that there was no significant difference in the clinical outcome of patient infected with 027 strains and

those infected with non-027 strains. Similarly, Karlowsky *et al.* (2012) reported that 91 % *C. difficile* hospital acquired infections were attributed to North America pulsed-field type 2 (NAP2) instead of the North America pulsed-field type 1 (NAP1) or ribotype 027 strains. On the contrary, a different study has shown that severe *C. difficile* infection was associated with NAP1/ribotype 027 strains (Miller *et al.*, 2010). The study showed that patients aged 60–90 years were approximately twice as likely to experience severe outcomes if their infection was due to NAP1 compared with infections due to other NAP types. In a similar report, CDI was compared between patients infected with strains possessing the *C. difficile* ToxA and ToxB in addition to the binary toxin and patients infected with ToxA and ToxB positive strains only. It was observed that there was a significant difference in case fatality rate between the two groups with the former group showing higher fatality rate than the latter (Bacci *et al.*, 2011). However, the higher fatality rate among patients infected with *C. difficile* strains possessing the binary toxin genes as well as ToxA and ToxB was found to be irrespective of their ribotypes (Bacci *et al.*, 2011). Therefore, Bacci *et al.* (2011) suggested that the binary toxins generally contribute to the pathogenicity of ribotypes which are positive for ToxA and ToxB and are associated with more severe CDI as previously reported (Barbut *et al.*, 2005, Geric *et al.*, 2006). Therefore, other markers of virulence in *C. difficile* 027 than ribotype, may be contributing to its pathogenicity and severe CDI (Wilcox and Spencer, 1992, Warny *et al.*, 2005, Makris and Gelone, 2007, Sun *et al.*, 2011, Oka *et al.*, 2012).

1.1.11.2 The *C. difficile* 027 subclades

C. difficile 027 exits in diverse subclades. Fawley *et al.* (2008) used multilocus variable-number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE) to identify 23 MLVA sub-types and 5 Pulsovar types within 91 clinical isolates

of *C. diffcile* 027 strains obtained from 9 hospitals in England (Figure 1.6; Figure 1.7). The MLVA procedure identified seven regions with short tandem repeats (A6, B7, C8, E7, F3, G8 and H9) within the genome of *C. difficile* and primers were designed to amplify them. The sequences of the PCR fragments were analysed using multicolored capillary electrophoresis on an ABI3100 genetic analyser, with a ROX500 marker as an internal marker for each sample. The repeat numbers were analysed using unweighted-pair group method with arithmetic mean clustering (UPMGA) and the multistate categorical similarity coefficient. Similarities between strains in at least 5 out 7 of the markers (71 % similarity) were grouped into a single subclade (MLVA type) (Figure 1.6). For the Pulsovar typing, PFGE was used to generate dendograms using UPMGA after macrorestriction endoclease digestion of the representative DNA with Sma1 for 5 h at 30°C. Similarities within strains by 98 % of the dendograms were assigned a Pulsovar (Figure 1.7) (Fawley *et al.*, 2008). In addition, the report of Fawley *et al.* (2008) indicated that high mortality was associated with specific MLVA or Pulsovar types. Since these strains belong to the same ribotype but differed in virulence, there could be other factors beside mutations in the negative regulatory gene and the binary toxin gene carriage that may be responsible for the observed differences in their pathogenicity. One possible factor that can cause this diversity is bacteriophage carriage.

A6	B7	C6	E7	F3	G8	H9	MLVA type	Institution	Hospital site	Isolate
34	18	16	11	4	9	2	1	A	1	14L
34	22	16	13	4	9	2	1	A	1	52L
22	22	16	13	4	9	2	1	A	1	38L
22	12	35	9	4	?	2	2	A	1	2L
38	20	16	12	4	10	2	3	C	7	12L
38	20	16	12	4	10	2	3	C	7	87L
39	20	16	12	4	10	2	3	C	7	10L
39	20	16	12	4	10	2	3	C	7	79L
35	20	16	12	4	10	2	3	C	8	28L
11	20	19	12	4	11	2	4	C	7	83L
11	20	19	12	4	11	2	4	C	7	92L
11	20	19	12	4	11	2	4	C	7	85L
11	20	19	12	4	11	2	4	C	7	88L
11	20	19	12	4	11	2	4	C	7	89L
11	20	17	12	4	11	2	4	C	7	29L
11	20	17	12	4	11	2	4	C	7	78L
24	20	46	10	4	11	2	5	A	1	32L
44	22	17	14	4	10	2	6	A	1	17L
24	22	17	9	4	10	2	6	A	1	81L
27	23	17	12	4	10	2	7	A	1	67L
27	23	17	12	4	10	2	7	A	1	61L
27	22	17	12	4	10	2	7	A	1	54L
27	22	17	12	4	10	2	7	A	1	75L
28	22	17	12	4	10	2	7	GP	GP	25L
28	22	17	12	4	10	2	7	A	1	77L
27	22	17	12	4	14	2	7	A	1	21L
27	22	18	12	4	4	2	8	A	1	5L
27	14	18	12	4	10	2	8	GP	GP	9L
31	21	22	12	4	11	2	9	B	5	26L
32	21	24	12	4	11	2	9	B	5	82L
32	21	17	12	4	9	2	10	A	3	94L
20	21	23	12	4	10	2	11	A	2	90L
30	21	23	12	4	10	2	11	B	5	86L
37	21	14	12	4	10	2	11	B	5	95L
34	21	20	12	4	10	2	12	GP	GP	60L
34	21	20	12	4	10	2	12	A	1	63L
34	21	20	12	4	10	2	12	A	3	65L
35	21	21	12	4	10	2	12	GP	GP	7L
34	21	17	12	4	10	2	12	A	2	66L
35	21	17	12	4	11	2	12	A	2	39L
33	21	21	12	4	10	2	13	B	5	22L
32	21	21	12	4	10	2	13	B	5	96L
32	21	22	12	4	10	2	13	B	6	6L
32	21	22	12	4	10	2	13	B	5	68L
32	21	22	12	4	10	2	13	B	5	93L
32	21	22	12	4	10	2	13	B	6	69L
32	21	22	12	4	10	2	13	B	6	73L
31	21	22	12	4	10	2	13	B	5	84L
33	21	22	12	4	10	2	13	B	6	4L
34	22	22	12	4	10	2	13	B	6	70L
32	22	22	12	4	10	2	13	B	5	80L
33	22	22	12	4	10	2	13	GP	GP	46L
37	19	35	10	4	11	2	14	A	1	23L
37	19	22	10	4	10	2	14	A	1	47L
37	19	28	10	4	9	2	14	A	3	57L
29	19	34	10	4	9	2	15	A	2	31L
29	19	34	10	4	9	2	15	A	2	33L
29	19	35	10	4	9	2	15	A	2	34L
29	18	36	10	4	9	2	15	A	1	59L
29	21	45	10	4	9	2	15	A	2	20L
28	20	44	10	4	9	2	15	A	2	91L
39	19	29	10	4	12	2	16	A	1	3L
39	18	25	10	4	12	2	16	A	1	55L
37	19	23	10	4	12	2	16	A	1	1L
37	19	23	10	4	12	2	16	A	1	42L
39	19	23	10	4	12	2	16	A	1	15L
39	19	23	10	4	12	2	16	A	1	16L
39	19	23	10	4	12	2	16	A	1	19L
39	19	23	10	4	12	2	16	A	1	30L
39	19	23	10	4	12	2	16	A	1	35L
39	18	23	10	4	12	2	16	A	1	56L
39	19	23	10	4	12	2	16	GP	GP	50L
39	19	23	10	4	12	2	16	GP	GP	76L
22	19	23	10	4	12	2	16	A	1	18L
9	18	23	10	4	12	2	16	A	1	43L
9	18	25	10	4	12	2	16	A	1	44L
36	18	36	10	4	10	2	17	A	1	49L
36	19	40	10	4	10	2	17	A	1	71L
36	18	35	10	4	10	2	17	A	1	58L
36	18	35	10	4	10	2	17	A	1	62L
35	19	35	10	4	10	2	17	A	1	64L
18	20	23	10	4	6	2	18	GP	GP	37L
18	18	23	10	4	6	2	18	GP	GP	51L
18	19	24	10	4	6	2	18	A	1	48L
19	18	31	10	4	2	2	19	GP	GP	13L
27	16	44	10	4	9	2	20	A	1	53L
26	16	44	10	4	9	2	20	A	1	45L
26	16	45	10	4	10	2	21	A	1	40L
27	21	39	10	4	9	2	22	A	4	36L
27	21	40	10	4	9	2	22	A	4	41L
19	10	26	10	4	13	2	23	D	9	72L

Figure 1.6 Dendogram showing the 23 different MLVA types of *C. difficile* 027 (Fawley *et al.,* 2008).

The dendogram of 91 *C. difficile* 027 isolates showing their relatedness within the 7 MLVA markers (A6, B7, C8, E7, F3, G8 and H9). The repeat numbers for each of the markers were shown. Similarities between strains in at least 5 of the 7 markers (71 % similarity) were grouped into an MLVA type.
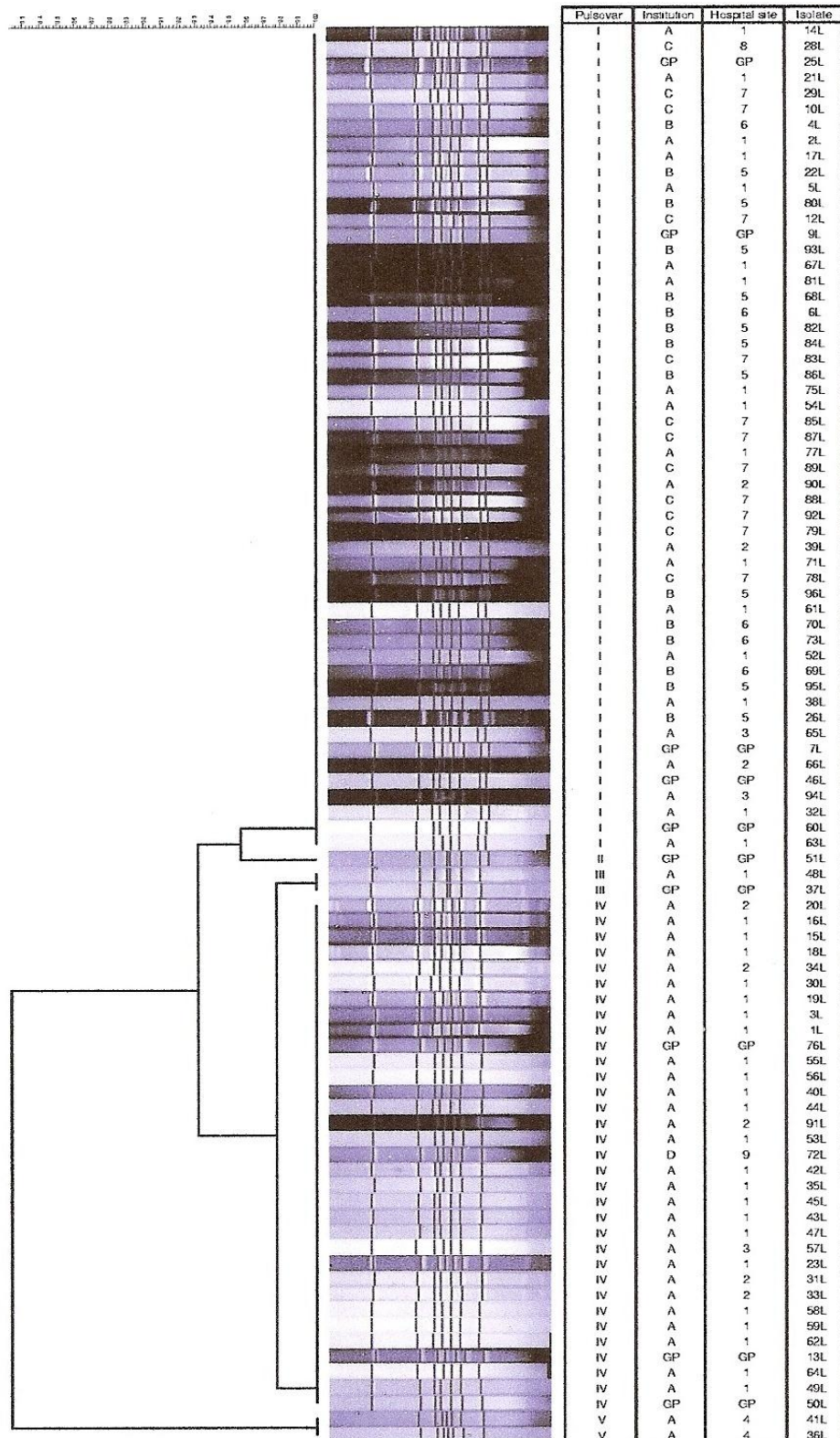
Figure 1.7 Dendogram of 91 isolates of *C. difficile* 027 showing their relatedness by macrorestriction endonuclease profile using PFGE. (Fawley *et al.*, 2008).

The DNA sampls of the 91 *C. difficile* 027 isolates were digested with Sma1 and then subjected to PFGE analysis. Dendograms generated from the PFGE were analysed using unweighted-pair group method with arithmetic mean clustering (UPMGA). Similarities within strains by 98 % of the dendograms were assigned a PFGE Pulsovar.

1.2   Bacteriophages

Bacteriophages (phages) are viruses that infect bacteria. Bacteriophages were jointly discovered by Frederick Twort (1915) in England and Felix d'Herelle (1917) at the Pasteur Institute in France (Pelczar *et al.*, 1988). Felix d'Herelle coined the term bacteriophage which means 'to eat bacteria'. They were so called because virulent bacteriophages can cause the compete lysis of a susceptible bacterial culture. Phages are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery (Cann, 1993, Summers, 2005). They occur widely in nature and can readily be isolated from sea water, faeces, sewage and in most places where their hosts are found (Abedon, 2006, Synnott *et al.*, 2009, Clokie *et al.*, 2010). Phages consist of a nucleic acid genome (DNA or RNA) surrounded by a protein coat called a capsid. Bacteriophages, like viruses, vary in their morphology. They may be icosahedral in shape consisting of 20 faces, filamentous or complex structures consisting of icosehedral heads with helical tails (Nicklin *et al.*, 1999). A typical T4 phage normally consists of an icosahedral capsid (head) which contains nucleic acid (commonly double stranded DNA), a tail of various lengths which has the tendency to contract and tail fibres which bind to the surface of their target bacteria (Figure 1.8) (Ackermann, 2003, 2006, Nolan *et al.*, 2006).
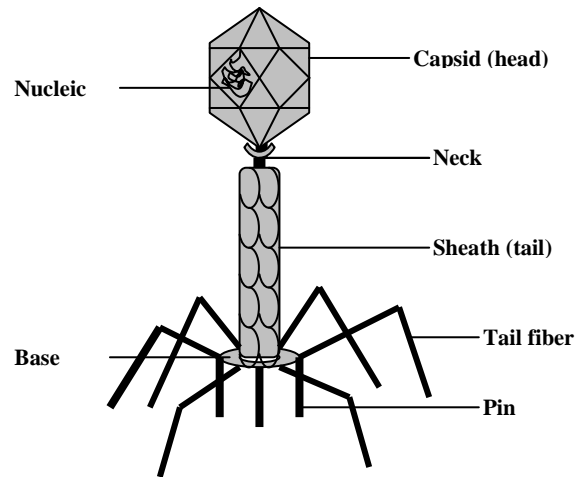
Figure 1.8 A diagram of a typical T4 phage.

1.2.1   Classification of bacteriophages

Bacteriophages are classified into 13 families according to their morphology, type of nucleic acid, and mode of replication (Ackermann 2006). Most phages have double stranded (ds) DNA, however, single stranded (ss) DNA, ds RNA and ss RNA also exist (Prescott *et al.*, 2002, Ackermann, 2006) (Figure 1.9).
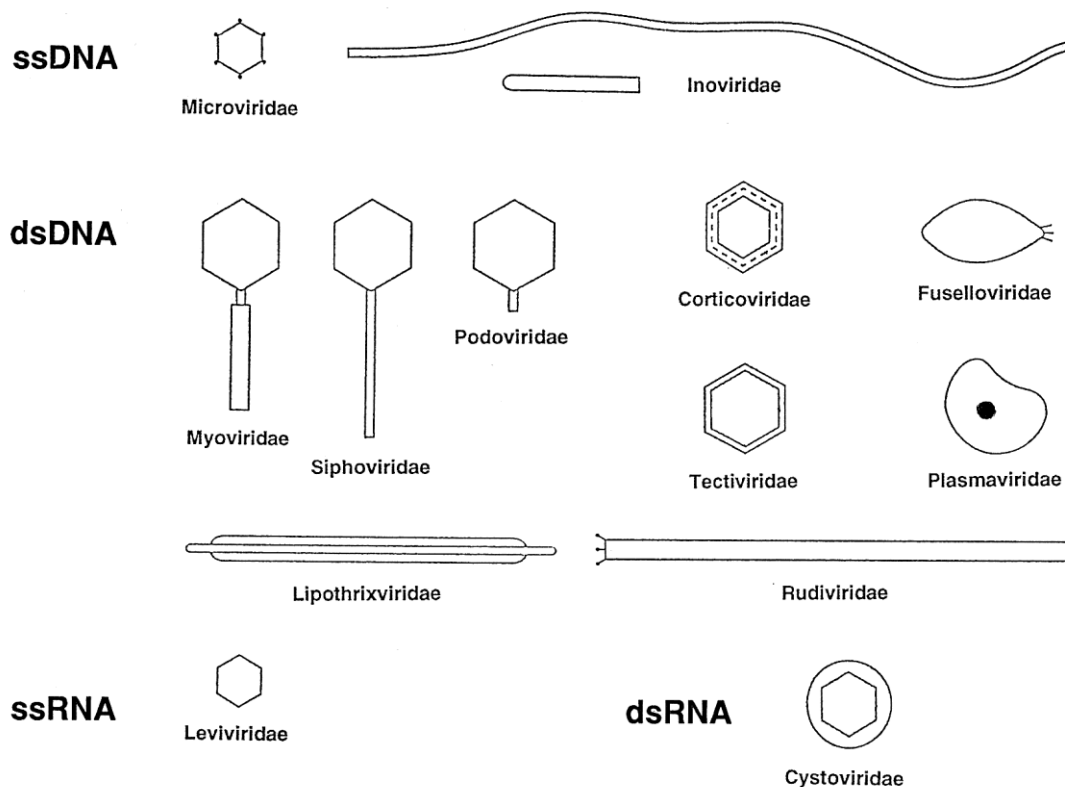
Figure 1.9 Diagram showing classification and morphologies of bacteriophage (Ackermann 2003).

Bacteriophages are classified into four major groups; the ssDNA, dsDNA, ssRNA and the dsRNA phages. The families *Myoviridae*, *Siphoviridae* and *Podoviridae* contain the dsDNA.

The tailed bacteriophages are the commonly known phages and consist of about 5,100 members (Ackermann, 2006). A tailed bacteriophage has a tail, an icosahedral head and double stranded DNA as its genome. The Baltimore classification identified seven classes of viruses. By this classification, tailed bacteriophages belong to Class I viruses and the order *Caudovirales*, which is further classified into three families according to the morphological features of the tail. The three families are: A, *Myoviridae* with contractile tails consisting of a sheath and a central tube; B, *Siphoviridae*, which consist of a long non-contractile tail; and C, *Podoviridae* with a very short and non-contractile tail (Figure 1.9) (Ackermann, 2003, 2006, Nolan *et al.*, 2006).

1.2.2 Life cycle of bacteriophages

The life cycle of a phage can either be a lytic or a lysogenic one (Sauvageau and Cooper, 2010). In the former, a series of events take place that lead to the death or lysis of the host, and in the latter, the phage does not necessary lyse the host but integrates and replicates its DNA as part of the host genome (Campbell, 2006). In either case, the life-cycle of a typical phage begins with adsorption; the process in which phages attach to the surface of a bacterium (Figure 1.10, Stage 1). The physiological condition of the bacterium determines the ability of a phage to adsorb to it (Delbrück, 1940, Prescott *et al.*, 2002). Attachment is enhanced by the ability of the phage adhesins to recognise specific binding sites known as receptors on the bacterium thus defining its host range. Receptors vary in form and nature and can either be protein, glycoprotein, phospholipids or lipopolysaccharide (Lindberg, 1973, Nicklin *et al.*, 1999, Rakhuba *et al.*, 2010). A successful attachment can lead to penetration (Figure 1.10, Stage 1)**.** Usually only the genetic material is injected into the host cell and the method through which this is done varies from one phage to another. In a typical myovirus, for example, which has a contractile tail, a complex mechanism is involved. The sheath contracts and the DNA is injected into the host cell. The empty phage, called the 'ghost', is left on the surface of the host cell. In other viral particles, translocation of the entire viral particle is mediated by proteins in the viral capsid and specific membrane receptors (Cann, 1993). Other viruses like influenza in the process of receptor-mediated endocytosis are taken in (Weissenhorn *et al.*). Once in the host, the phage genome may be damaged by host restriction endonucleases as part of its restriction-modification defence mechanism. The restriction enzymes bind to sequence-specific sites on the foreign DNA and cut it at these sites (Nicklin *et al.*, 1999). The phage DNA can sometimes be modified by methylation to protect them from the host restriction enzymes. DNA methylation involves the modification of the primary sequence of a gene by the addition of a methyl

group to the DNA nucleotide through the action of a methyl transferase enzyme (Enikeeva *et al.*, 2010). Bacterial DNA can also be modified this way to protect its native DNA from incoming bacteriophage (Reisenauer *et al.*, 1999, Kobayashi, 2001, Dong *et al.*, 2010). The T-even phages modify their DNA by either methylation or glycosylation to escape digestion by the host restriction enzymes (Medoff and Swartz, 1969, Hattman, 1970). The next step in the phage life cycle is nucleic acid replication (Figure 1.10, Stage 2). Once a phage successfully injects its DNA into the host, it may be transcribed and/or translated depending on the nature of the genetic material. This normally happens with the lytic phages. The lytic phages can switch off the replicatory mechanisms of their host to direct all resources for their own replication. This leads to the production of large amounts of nucleic acids, structural proteins and scaffolding proteins necessary for assembly, lysis and release. Once there are enough synthesised phage materials, the phage particles begin to assemble and the nucleic acid is packed into the capsid (Figure 1.10, Stages 3A and 4A) (Nicklin *et al.*, 1999). This is followed by the release of phage particles in case of lytic phages (Figure 1.10, Stage 4A). In the case of lysogenic or temperate phages, the phage DNA does not begin to replicate immediately, but is integrated into the chromosome of the host and replicates within the host chromosome (Figure 1.10, Stages 3B and 4B). The prophages (phages that are integrated into the genome of bacterial hosts) can be induced to enter into a lytic cycle, replicate and release phage progeny (Figure 1.10, Stage 5). The prophage induction may be spontaneous or induced using stress, uv radiation or antibiotics (Lydersen and Pollard, 1975, Barnhart *et al.*, 1976, Paolo, 1982, Choi *et al.*, 2010). The release is normally aided by the phage enzyme holin, which degrade the cell wall to release the phages (Wang *et al.*, 2000, Grundling *et al.*, 2001, Krupovic and Bamford, 2008).
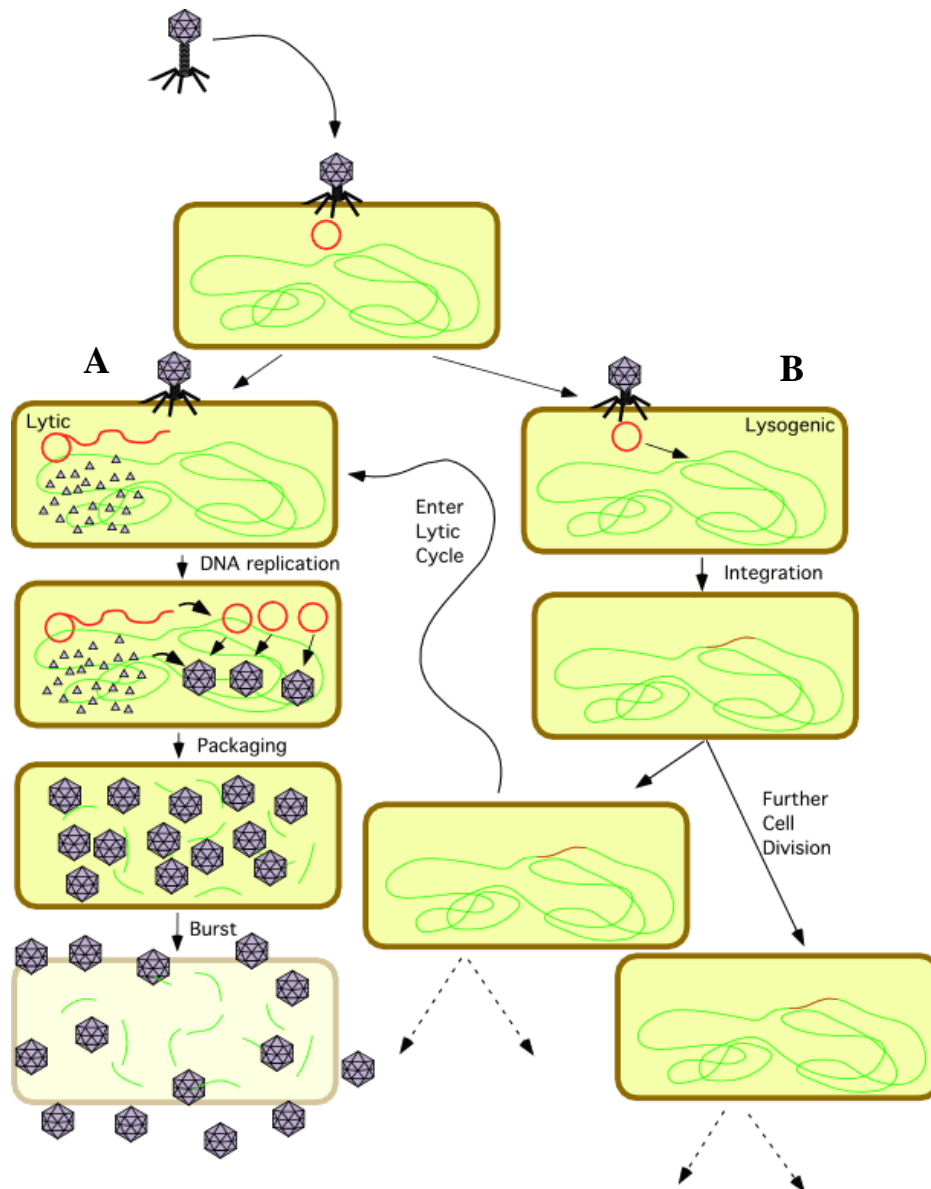
Figure 1.10 Diagram showing the life cycle of a typical bacteriophage.

The lytic (A) and lysogenic (B) pathways of phage replication are indicated.

1.2.3 Phages as agents of horizontal gene transfer

Generally, bacteria interact with phages in a number of ways. In the case of lytic phages, they disrupt bacterial metabolism and cause the bacteria to lyse releasing carbon and nutrients, which may influence bacteria growth (Sulakvelidze *et al.*, 2001). Phages with a lysogenic life-cycle can encode toxins that may influence the pathogenicity of their host and play an important role in the evolution of strains by

providing a mechanism for horizontal gene transfer between strains via transduction (James *et al.*, 2001, Saunder *et al.*, 2001). During lysogeny, temperate bacteriophages integrate their genome into the bacterial chromosome. Thus, the prophage constitute in many bacteria a substantial part of laterially acquired DNA (Canchaya *et al.*, 2003). Genomic studies has shown that a substantial part of the bacterial DNA is not acquired via vertical transfer (from parent cell to descendant) but through lateral gene transfer by transformation, conjugation or transduction (Bushman, 2002).

Horizontal gene transfer is widely studied in bacteria. In *Pseudomonas aeruginosa*, two phage-tail gene-clusters have developed into bacteriocin which are antagonistic to related bacteria thus, enhance survival by reducing competition for space and nutrients (Nakayama *et al.*, 2000). The prophages of *Bacillus substillis* and *Rhodobacter capsulatus* act as gene transfer agents of 13 kb and 4.5 kb fragments of bacterial DNA respectively (Okamoto *et al.*, 1968, Lang and Beatty, 2001, Fogg *et al.*, 2011). Similarly, in *Salmonella typhimurium* phage P22 or coliphage Mu occasionally package the bacterial DNA instead of phage DNA. Upon infection of the next host, this bacterial DNA can be incoparated into the bacterial chromosome (Kwoh and Kemper, 1978, Fogg *et al.*, 2011). In addition, phage mediated lateral gene transfers between *Streptococcus dysgalactiae* (Group G *Streptococcus,* GGS) and *Streptococcus pyogenes* (Group A *Streptococcus,* GAS) has been reported (Davies *et al.*, 2006). Toxin production by *Corynebacterium diphtheriae* is mediated by a gene carried by a beta phage and only those strains that have been converted by lysogeny are pathogenic (Welkos and Holmes, 1981).

Besides dissemination of genes to bacteria, phages plays significant role in the the emergence of new virulent bacteria strains particularly if the genes transferred are responsible for virulence (Canchaya *et al.*, 2003, Campbell, 2006). Besides increasing

virulence, phages have the ability to convert non-pathogenic strains into pathogenic ones not only as fragmented genes but as a whole phage. Such example can be found in the case of verocytotoxigenic *E. coli* (VTEC) where the VT genes are carried on temperate bacteriophages (VT phages (James *et al.*, 2001). Certain bacteria such as *Vibrio cholerae,* Shiga toxin-producing *E. coli*, *C. diphtheriae,* and *C. botulinum* depend on a specific prophage-encoded toxin to cause disease (Saunder *et al.*, 2001, Brussow *et al.*, 2004). In addition, phages can confer fitness to their lysogens thus increase their survival rate (Brussow *et al.*, 2004).

1.2.4 Bacteriophages of *C. difficile*

In *Clostridia* species, mobile genetic elements like bacteriophages and plasmids have played significant roles in the phenotypic characteristics of their hosts (Iida *et al.*, 1974, Bruggemann, 2005). Temperate bacteriophages of *C. difficile* can contribute to the pathogenicity of their host by differentially regulating the expression of bacterial toxins (Goh *et al.*, 2005a, Govind *et al.*, 2009, Sekulovic *et al.*, 2011). However, before any link between *C. difficile* pathogenicity and bacteriophages can be investigated, it is necessary to establish how widespread and diverse the phages are in clinical strains.

The first set of temperate bacteriophages to be induced from *C. difficile* was reported in 1983 (Sell *et al.*, 1983). They induced prophages from 254 clinical *C. difficile* isolates using mitomycin C at 3 μg/ml final concentration. Electron microscopy analysis conducted on the induced lysates revealed a virion with a contracted tail sheath. The virion was identical to other previously characterised Clostridia phage present in many mitomycin C induced lysates. In addition, Sell *et al*. (1983) reported the presence of bacteriocins in 20 of their induced filtrates. The bacteriocins were reported to be to able to clear lawns of susceptible *C. difficile* hosts without distinct plaque formation. The report also showed that induced filtrates from isolates obtained

from the same patients showed similar sensitivity patterns. However, induced lysates from patients' isolates from different geographical locations showed varied sensitivity patterns. Thus, their report was the first to show that phages can be used as a typing tool for *C. difficile*. Subsequently, two morphologies of tailed temperate *C. difficile* phages (a siphovirus and myovirus) were isolated and characterised (Mahony *et al.*, 1985). The phages may be spontaneously released or can be induced by uv light. Mahony *et al.* (1985) showed that the phages differ also in their physiology and host range. Similarly, Nagy and Foldes (1991) reported morphologically distinct phages after prophage induction of *C. difficile* isolates using mitomycin C. Further evidences of diverse temperate bacteriophage carriage in *C. difficile* were also reported (Goh *et al.*, 2005a, Goh *et al.*, 2005b, Fortier and Moineau, 2007, Goh *et al.*, 2007, Nale *et al.*, 2012). Goh *et al*. (2005a) reported the molecular relationship of the phages through partial DNA sequencing. The report also showed the influence of temperate bacteriophage carriage to ToxB production but the relationship between phage carriage and the pathogenicity locus (PaLoc) could not be ascertained (Goh *et al.*, 2005a). Although Goh *et al*. (2005a) were able to identify *tcdE*, a holin-like gene in three of the phages examined; they could not identify the *tcdA* and *tcdB* genes responsible for *C. difficile* ToxA and ToxB production. In addition, they confirmed through RT-PCR that the transcription of tcdC was not significantly altered by the infection of these phages on the lysogens. A similar study further supports the influence of phage infection on toxin production (Sekulovic *et al.*, 2011). Sekulovic *et al*. (2011), characterised phiCD38-2, a temperate *C. difficile* siphovirus with a relatively wide host range. They reported that infection of *C. difficile* strains with phiCD38-2 phage led to a 6-fold increase in toxin production. However, just like Goh *et al.* (2005a), they could not identify any virulence factor or *C. difficile* toxin genes within the genome of phiCD38-2.

Another *C. difffficile* phage, a myovirus (phiCD27) was isolated and characterised and the purified phage endolysin was shown to lyse other *C. difficile* strains including ribotype 027 (Mayer *et al.*, 2008). Thus, the endolysin could be harnessed for therapeutic purposes.

The first report on temperate bacteriophage carriage on *C. difficile* ribotypes was reported in 2007. Tailed temperate bacteriophages were induced from six different *C. difficile* ribotype strains (Fortier and Moineau, 2007). The report showed that each ribotype harboured a specific phages identified by their morphologies and restriction profiles. Therefore, it was suggested that ribotypes were indicators of their prophage content. The report of Fortier and Moineau (2007) was thought to be significant as it was the first to show phage carriage in relation to *C. difficile* ribotypes. However, the selection of isolates from the same outbreak strains and small samples size were great limitations to the work. Prior to my findings, reports on phage carriage on ribotype 027 were grossly insufficient as only one phage morphology (a myovirus) was identified from a single strain of this ribotype. My MRes and current research examined 16 and 91 isolates of ribotype 027 respectively and found that they contain morphologically and genetically distinct set of temperate bacteriophages that contribute to diversity within this ribotype (Nale, 2009, Nale *et al.*, 2012).

Prevalence of prophage carriage in genomes of *C. difficile* strains has been reported to be widespread but only few inducible phages have been reported (Henn *et al.*, 2010). Goh *et al.* (2007) investigated the prevalence of *C. difficile* prophages in clinical isolates using the myovirus phiC2 as a genomic probe in dot blots and Southern blots. They were able to detect prophages in 31 of the 37 clinical isolates (Goh *et al.*, 2007). In another report, when genomes of 25 *C. difficile* 027 strains were analysed, 22 of the strains were found to contain prophages but the prophages were not characterised

further (Henn *et al.*, 2010). Surprisingly, when 56 *C. difficile* isolates were induced from a different study, only three of the strains yielded phages when induced with mitomycin C (Goh *et al.*, 2005b). Similarly, only two phages were observed when 96 *C. difficile* clinical strains were induced in another study (Mahony *et al.*, 1985). Therefore, the few previously characterised temperate bacteriophages in *C. difficile* only represent a small fraction of phages that may be present in clinical and environmental strains (Henn *et al.*, 2010, Nale *et al.*, 2012). The scarcity of information on inducible phages of *C. difficile* may be attributed to the choice of inducing agent and the inability of the TEM analysis to detect phages in the induced lysates due to very low phage titer. In addition, the limitation of finding a suitable host for the propagation of these phages is a factor that limits the study of *C. difficile* phages (Nagy and Foldes, 1991, Nale *et al.*, 2012). Consequently, there is a need to develop molecular based methods that are culture independent and can detect *C. difficile* phages *in-situ*. This alternative method will greatly aid morphological studies and direct strain choice before the labour intensive prophage induction procedures and transmission electron microscopy analysis.

Molecular markers for phage identification will rely on conserved genes in the phage genomes. Five temperate *C. difficile* phages have been fully sequenced and annotated. Three belong to the *Myoviridae* (phiC2, phiCD119, and phiCD27) and two to the *Siphoviridae* (phiCD6356 and CD38-2) (Govind *et al.*, 2006, Goh *et al.*, 2007, Mayer *et al.*, 2008, Horgan *et al.*, 2010, Sekulovic *et al.*, 2011). The alignment of *C. difficile* myovirus genomes showed that genes such as those that encode for the capsid, portal and holin proteins have nucleotide homology (Goh *et al.*, 2007). Prior to this study, no report has previously used these genes in order to design molecular markers or establish phylogenetic relationships within *C. difficile* ribotypes or strains. In this

study, three molecular markers targeting the capsid, holin and portal genes specific to *C. difficile* phages have been designed (Nale *et al.*, 2012, Shan *et al.*, 2012). These markers have the potential to detect and predict phage presence in clinically relevant strains of *C. difficile*. These markers are discussed further in section 4 of this thesis.

1.3   Justification of study

Although the incidence of CDI has been reported to fall in few regions in the UK since 2008/09, the number of fatal cases still remains significantly high and worrying in many parts of the UK and in the industrialised world in general. *C. difficile* 027 is diverse and remains the most clinically relevant ribotype responsible for more than 20 % of fatal CDI cases in the UK. There is therefore a need to define role of factors that may be contributing to the diversity and severity of CDI caused by *C. difficile* 027 strains. The factor investigated in the study is temperate bacteriophage carriage. Recent genomic analysis has identified mobile genetic elements including prophages in 22 out of 25 *C. difficile* 027 strains, but the bacteriophages were not characterised further (He *et al*. 2010). In another study, when a *C. difficile* 027 outbreak strain was investigated, only single bacteriophage morphology was observed (Fortier and Moineau 2007). No previous studies have examined bacteriophage carriage within the subclades of *C. difficile* ribotype 027. To determine if bacteriophages are contributing to the diversity within *C. difficile* 027 strains, the range of temperate bacteriophage types associated with ribotype subgroups must first be established. Also, there is need to design molecular markers for the identification of *C. difficile* phages which will greatly enhance physiological and morphological studies. In addition to phage carriage, sporulation in *C. difficile* 027 strains was also examined. The *C. difficile* endospores provide protection against disinfection and cleaning thereby making the environment a reservoir for re-infection. Few reports have compared sporulation rates among 027

strains but these have not been correlated to their disease severities or prophage content (Wilcox and Fawley, 2000, Akerlund *et al.*, 2008, Burns *et al.*, 2010a, Burns *et al.*, 2011, Heeg *et al.*, 2012). In this study, sporulation rates among *C. difficile* 027 isolates were examined and these were correlated to their diverse subclades and prophage content.

1.4   Research questions

1. Do all *C. difficile* 027 strains contain bacteriophages?

2. If so do they carry the same or different phages?

3. Is there a correlation between temperate bacteriophage carriage and *C. difficile* 027 subclades?

4. Can phage genes be used as molecular markers to characterise *C. difficile* phage presence and diversity?

5. Do all *C. difficile* 027 strains have the same sporulation characteristics?

6. Is there a correlation between sporulation rates and the ribotype 027 subclades and phage carriage?

1.5   Aims of study

The aims of this study were to:

1. induce and characterise temperate bacteriophages from 91 *C. difficile* 027 subtypes' clinical isolates obtained from 9 hospitals in England.

2. correlate phage carriage to the *C. difficile* 027 subclades and to ascertain if phage carriage may contribute to the diversity and their consequent disease severities.

3. design molecular markers to examine phage diversity.

4. determine sporulation rates among *C. difficile* 027 subclade strains and correlate this to their diverse disease severities and phage carriage.

2 Materials and methods

This section describes all the general protocols and reagents used in this study.

2.1 Media, buffers and solutions

All reagents, buffers and solutions used in this study are described in Appendix 1. *C. difficile* media were pre-reduced anaerobically at 37 $^{\circ}$C for at least 1 h prior to use. This procedure was necessary to pre-warm the media and eliminate oxygen from them.

2.2 Bacterial isolates and phages

There were two sets of *C. difficile* isolates used in this study. The first set was a collection of 91 ribotype 027 isolates which were the strains used to study phage diversity and sporulation. The second set consisted of representative isolates of ribotypes 001, 002, 005, 012, 014, 015, 020, 078 and 220 used as tools on which to study the molecular diversity of *C. difficile* phages, plaque assays and spot tests. *Escherichia coli* DH5α-T1 (chemically competent cells) were used for cloning. Phage phiCD27 was kindly donated by Dr M. J. Mayer (Norwich Research Park, Norwich, UK) and S-PM2 from this laboratory. The DNA samples of phiCD27 and S-PM2 were used as controls to optimise the PCR procedures for the capsid, holin and portal protein primers.

2.2.1 Source of the *C. difficile* 027 isolates

The 91 *C. difficile* 027 isolates were collated by Prof. M. H. Wilcox (University of Leeds, UK). They were isolated from *C. difficile* toxin positive faecal samples that were submitted as part of routine diarrhoea surveillance in nine hospitals in England. The isolates had previously been categorised into 23 and 5 subtypes using multilocus

variable-number tandem-repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE), respectively (Fawley *et al.*, 2008). The isolates were received in form of spores embedded on filter paper discs.

2.2.1.1 Isolation of *C. difficile* from filter paper discs impregnated with spores

Spores were recovered from the paper discs by streaking them onto Brazier's selective growth medium also known as cefoxitin cycloserine egg yolk agar (CCEY agar) (Bioconnections, Leeds, UK) (George *et al.*, 1979, Clabots *et al.*, 1991, Kato *et al.*, 2010). The spores were incubated in an anearobic chamber (10 % H_2, 5 % CO_2, and 85 % N_2) (Don Whitley Scientific, West Yorkshire, UK) at 37 ^{o}C for 48 h. Following incubation, single bacterial colonies were picked using sterile plastic loops and streaked on pre-reduced non-selective brain heart infusion (BHI) agar plates supplemented with 7 % defibrinated horse blood (Oxoid, Hampshire, UK) and incubated anaerobically. *C. difficile* colonies were identified on BHI blood plates after 48 h incubation. Identification was based on their characteristic colony morphology (Figure 2.1), horse manure smell due to *p*-cresol production and yellow-green fluorescence under the long wave UV light. In addition, agglutination using a latex agglutination test specific for *C. difficile* cell wall antigens was used to confirm the *C. difficile* colonies (Oxoid, Hampshire, UK, Figure 2.2) (Shahrabadi *et al.*, 1984, Fille *et al.*, 1998, Kato and Kato, 1998).
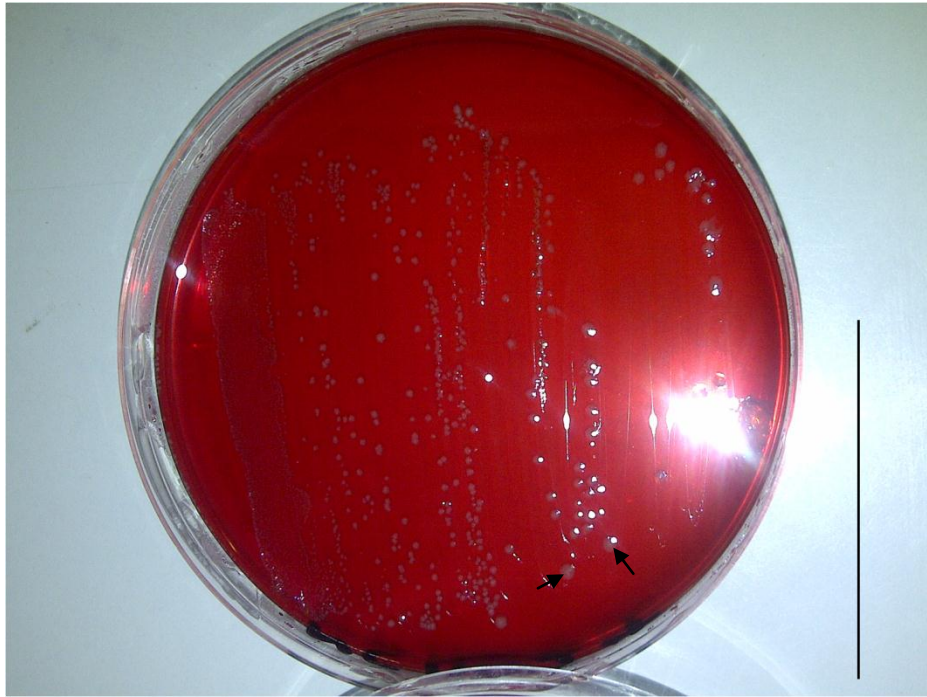
Figure 2.1 Picture showing characteristic colony morphology of *C. difficile* after 48 h growth on BHI 7 % blood medium. Bar represents ~5 cm.

For each isolate, a distinct *C. difficile* colony growing on Brazier's cefoxitin cycloserine egg yolk (CCEY) agar was streaked on Brain Heart Infusion (BHI) agar plate supplemented with 7 % defibrinated horse blood and cultured anaerobically for 48 h**.** Positive *C. difficile* colonies on the BHI plates are grey, opaque and non-haemolytic about 3-5 mm in diameter with irregular, lobate or rhizoidal edges as indicated by the black arrows in the picture above.
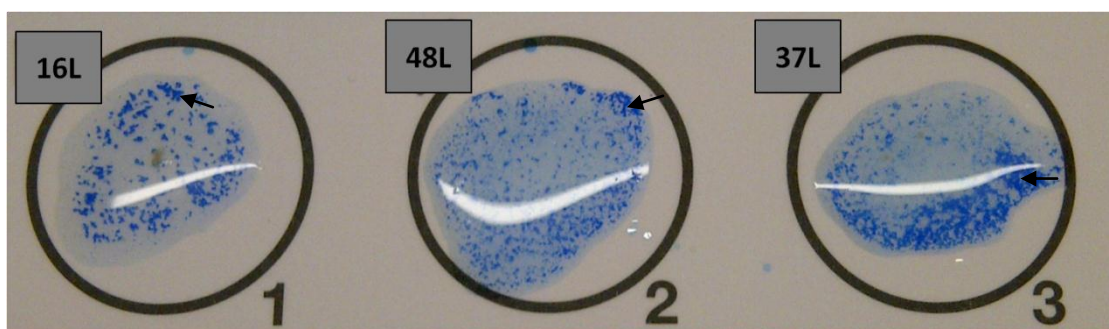
Figure 2.2 Picture showing positive latex agglutination test for three *C. difficile* 027 isolates (16L, 48L and 37L) used in this study.

A pure colony of *C. difficile* growing on BHI 7 % blood agar plate was identified using a latex agglutination test (Oxoid, Hampshire, UK). A positive agglutination shows blue precipitation (indicated by arrows) and occurs within 2 min as seen in samples 16L, 48L and 37 L above.

2.2.2 Sources of other *C. difficile* ribotypes isolates

The *C. difficile* reference strain CD630 (ribotype 012) strain was kindly provided by Prof. Neil Fairweather (Imperial College, London, UK). *C. difficile* strain NC11204 (ribotype 001) was obtained from the National Collection of Type Cultures (NCTC) (Central Public Health Laboratory, London, UK). Other *C. difficile* ribotype isolates including 002, 005, 014, 015, 020, 078 and 220 were isolated and ribotyped from *C. difficile* toxin A and B positive faecal samples collected from patients at the University Hospitals of Leicester (UHL) (Nale, 2009).

2.2.2.1 Isolation of *C. difficile* from faecal samples

Fresh faecal samples from diarrhoeal adult patients in UHL were collected between the periods of June 2007 and January 2009. The stool samples were tested positive for *C. difficile* toxin A and or B using an enzyme-linked immunosorbent assay (ELISA) for the detection of *C. difficile* toxins A and B (*C. difficile* Tox A/B // ™, Techlab, U.S.) (George *et al.*, 1982, Popoff, 2003, Pituch *et al.*, 2005, Musher *et al.*, 2007, Indra *et al.*, 2008, Kufelnicka and Kirn, 2011). The samples were collected into screw-capped polystyrene vials fitted with plastic spoons. They were transported on ice and on reaching the laboratory were immediately refrigerated at 4°C. Isolation of *C. difficile* was carried out immediately as previously described (Borriello and Honour, 1981). Briefly, the samples were subjected to alcohol shock by adding equal volume of industrial methylated spirit to it. This procedure helped to eliminate other bacterial contaminants and caused *C. difficile* vegetative cells to sporulate (Shahrabadi *et al.*, 1984). The faecal mixture was vortexed and incubated for 2 h at room temperature. *C. difficile* was isolated and identified from this mixture using Brazier's selective medium and BHI medium supplemented with 7 % horse blood medium as described in section 2.2.1.1.

2.3   Polymerase chain reaction (PCR) ribotyping

2.3.1   Bacterial DNA isolation using Chelex-100 solution

DNA was extracted from identified *C. difficile* colonies using Chelex® 100 Molecular Biology Grade Resin (BioRad Laboratories, Carlifornia, USA) (Bidet *et al.*, 2000, Arroyo *et al.*, 2005, Marsh *et al.*, 2006, Peterson *et al.*, 2007). To do this, 5 % (w/v) Chelex-100 was prepared in ultra pure water and 150 µl was dispensed into an eppendorf tube. A loop-full of *C. difficile* colonies from a 48 h culture on BHI blood plate was added. This was done for all the samples. The bacterial mixtures were vortexed and heated at $100^{o}C$ for 10 min. To prevent accidental opening of the lids due to mounting pressure, the eppendorf tubes were secured with fasteners during the heating process. After cooling for 5 min, the mixtures were centrifuged at $2,100 \times g$ for 10 min. The supernatants containing the DNA templates were collected into sterile eppendorf tubes and used immediately for PCR ribotyping or stored at $-20^{o}C$ for a maximum of two weeks.

2.3.2   Bacterial DNA isolation using phenol/ chloroform/ isopropanol method

An inoculum of *C. difficile* was prepared by transferring a loop-full of colonies from a 48 h culture on BHI blood medium into 2 ml pre-reduced fastidious anaerobe broth (FAB) (Bioconnections, Leeds, UK) (Alfa *et al.*, 2002, Rotimi *et al.*, 2003). The culture was allowed to grow for 18-24 h anaerobically. A 5 ml BHI broth was inoculated with 10 % of the inoculum. Following 10-15 h incubation, *C. difficile* cells from the BHI broth were harvested by centrifuging at $15,000 \times g$ for 5 min. Bacterial DNA was extracted using phenol/ chloroform/ isopropanol as previously described (Barbut *et al.*, 1993). Briefly, bacterial cell pellet was mixed with 500 µl of 0.1 x sodium saline citrate (SSC) buffer and transferred to an eppendorf tube followed by

centrifugation at 15,000 × g for 5 min. The resulting pellet was re-suspended in 300 µl of 10 mM Tris.Cl pH 8.0 containing lysosyme to 2.5 mg/ml and incubated at 37 ^{o}C for 30 min. Afterwards, the cells were lysed with 500 µl of bacterial lysis buffer containing 0.2 mg/ml of proteinase K. To allow complete lysis of cells, the mixture was further incubated at 37°C for 30 min before being centrifuged at 15,000 × g for 5 min. The supernatant was collected into a sterile eppendorf tube. To denature and precipitate protein contaminants, the supernatant was washed with an equal volume (0.8 ml) of phenol/chloroform/isopropanol (25:24:1 v/v/v). The solution was well mixed, incubated for 2 min at room temperature before being centrifuged at 15,000 × g for 5 min. The aqueous layer containing nucleic acids was gently recovered being careful to avoid the interphase. This solution was then mixed again with equal volume of chloroform/isopropanol (24:1 v/v), incubated at room temperature for 2 min and centrifuged at 15,000 × g for 5 min. The aqueous layer was recovered into a clean eppendorf tube and DNA was precipitated from it by adding 2 volumes of isopropanol and 0.4 volume of 7.5 M ammonium acetate. After mixing by gentle inversion, the DNA material was centrifuged down at 21,000 × g for 30 min at 4 ^{o}C. After removing the supernatant, the resulting DNA pellet was air-dried for 5 min before washing once with 1 ml of 75 % ethanol. Finally, the DNA was recovered by final centrifugation at 21,000 × g for 15 min. The pellet was again air dried for 5 min and re-suspended in 50 ml 10 mM Tris.HCl, pH 8.0. A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to analyse the quantity and quality of the DNA samples.

2.3.3 Ribotyping procedure

The *C. difficile* forward and reverse primer sequences, cdiffFP3 5'-CTGGGGTGAAGT CGTAACAAGG -3' and cdiffRP4 5'

GCGCCCTTTGTAGCTTGACC -3' respectively were used for PCR ribotyping (Stubbs *et al.*, 1999). A total of 50 µl volume PCR reactions were performed with SensoQuest LabCycler (SensoQuest GmbH, Germany). The 50 µl PCR reaction mixtures contained 0.5 µM of each of the primers, 0.25 mM of dNTPs, 1 x PCR buffer (Bioline, London, UK), 0.1 U of *Taq* polymerase (Bioline, London UK) and 2 µl of bacterial Chelex prepared DNA template except for the negative control where equal volume of ultra pure water replaced the template. HyperLadder IV (5 µl) (Bioline, London, UK) was used as a molecular standard.

The PCR ribotyping conditions were: denaturation at 95 $^{\circ}$C for 120 s followed by 30 cycles denaturation at 92 $^{\circ}$C for 60 s, annealing at 55 $^{\circ}$C for 60 s, elongation at 72 $^{\circ}$C for 90 s and a final extension at 72 $^{\circ}$C for 5 min. The reaction mixture was reduced in volume to ~20 µl on a heating block set at 75 $^{\circ}$C. PCR products may be kept at -20 $^{\circ}$C until needed or be immediately resolved in 1 % w/v RESponse Regular PCR Agarose gel (Bioplastics, The Netherlands) prepared in 1×Tris-acetate-EDTA (TAE, pH 8) containing 0.1 µl/ml GelRed (Biotium, Hayward, California, USA) (Nale *et al.*, 2012). The gel was run in TAE buffer at 90V for 4 h (150 mA). Gels were analysed using Genesnap software (SynGene) in the UV transilluminator. *C. difficile* ribotypes were determined by comparing PCR ribotype profiles produced to those of known profiles (obtained from *C. difficile* Ribotyping Network for England and Northern Ireland reference laboratory at Leeds).

2.4   Preservation of *C. difficile*

The *C. difficile* isolates were preserved in meat broths and in Protect Bacterial Preservers (Asha and Wilcox, 2002, Sharp *et al.*, 2010). A single *C. difficile* colony of isolates from a 48 h culture on blood agar medium was inoculated into BHI meat broths (Bioconnections, Leeds, UK). This was followed by incubation anaerobically at 37 $^{\circ}$C for

24 h with the caps slightly loosened. After incubation, the caps of the culture containers were tightened, sealed with parafilm and stored at room temperature for maximum period of six months. To cryopreserve *C. difficile*, a single colony was inoculated into 2 ml of FAB medium and incubated overnight. Approximately 1 ml of the sample was placed in an eppendorf tube and centrifuged at room temperature for 10 min at 2,100 × *g*. The supernatant was discarded and pellet was resuspended with the glycerol solution sucked out from the Protect Bacterial Preservers vial (Technical Service Consultants Limited, Heywood, UK). The glycerol and pellet mixture was then transferred from the eppendorf to the vial and was immediately placed in a -80 °C freezer for long time storage.

2.5   Phage isolation and purification

2.5.1 Prophage induction

Prophages were induced from the 91 *C. difficile* isolates using norfloxacin or mitomycin C (Matsushiro *et al.*, 1999, Fortier and Moineau, 2007, Nale *et al.*, 2012). Ten isolates were selected for the optimisation of the prophage induction procedure. Starter cultures of the isolates were produced as described in section 2.3.2. A 10 ml pre-reduced BHI broth was inoculated with 10 % of the starter culture and incubated anaerobically at 37 °C overnight or until an OD_{550} of 0.7- 0.9 was achieved. Each culture was treated with either norfloxacin (Sigma-Aldrich, Dorset, UK) or mitomycin C (Fisher Scientific, Loughborough, UK) at a final concentration of 0.3, 1, 3, 6 or 9 μl/ml with shaking at 100 rpm for 24 h. The OD_{550} was also taken at the end of the induction process. The induced cultures were centrifuged at 2,100 × *g* for 10 min and the resulting supernatants were filtered through a 0.22 μm filters and used for PEG purification and transmission electron microscopy (TEM) or kept at 4 °C until needed.

Having determined the optimal induction condition for the isolates, all 91 isolates were induced with norfloxacin or mitomycin C at 3 µl/ml final concentration for 24 h (Nagy and Foldes, 1991, Fortier and Moineau, 2007, Nale *et al.*, 2012). The OD_{550} was taken before and at the end of the 24 h inductions at this concentration. To determine the 120 h effect of mitomycin C or norfloxacin induction on *C. difficile* cultures and to correlate this to prophage release, the induction time of 15 selected cultures were allowed up to 120 h. The OD_{550} was taken at additional time points of 48, 72, 96 and 120 h following induction with norfloxacin or mitomycin C. The experiment was terminated after 120 h. At the various time points, approximately 1 ml samples were taken from the induced cultures. The samples were centrifuged and filtered as above and used for TEM analysis.

2.5.2 Phage purification using polyethylene glycol

The induced phage supernatants were purified using a solution of polyethylene glycol (PEG) and NaCl (Yamamoto *et al.*, 1970, Goh *et al.*, 2005b). The solution of PEG and NaCl enable the bacteriophages to be pelleted down during centrifugation. The resultant pellet was resuspended in a small amount of phage buffer, resulting to ~100-fold concentration of the original the induced lysate (Yamamoto *et al.*, 1970). To purify the phage supernatant using PEG, NaCl was added to 1 M, mixed and incubated on ice for 1 h and then centrifuged at $10,000 \times g$ for 5 min. Then 10 % (w/v) of PEG 8000 (Fisher Scientific, New Jersey, USA) was slowly added and continuously stirred until completely dissolved. This was kept at 4 ^{o}C overnight and afterwards centrifuged at $15,000 \times g$ for 20 min at 4 ^{o}C to precipitate the phage. The supernatant was discarded and the pellet was re-suspended in 1 ml of SM buffer (10 mM NaCl, 8 mM $MgSO_4.7H_2O$ and 50 mM Tris-Cl), washed with equal volume of chloroform and

centrifuged for 15 min at 5,000 × g. The top phase was collected and filtered using membrane filter of pore size 0.22 μm. The filtrate was used for TEM analysis.

2.6 Characterisation of phages

2.6.1 Transmission electron microscopy (TEM)

TEM was used to identify the phage morphologies (Biller *et al.*, 2005, Goh *et al.*, 2005b, Shan *et al.*, 2012). To do this, 300-mesh copper grids were coated with thin film of pioloform which acts as a base for the carbon to be applied to. The pioloform coated copper grids were allowed to air dry for 1 h and were then stabilised by applying a thin coating of carbon. The grids were made hydrophilic side up by high voltage glow discharge with argon gas for 30 s. About 5 μl of the phage sample was immediately deposited on the carbon side of a coated grid and allowed to stand for about 5 min. Thereafter, excess sample was removed by blotting with Whatman paper. The grid was rinsed with two to three drops of double distilled water to remove buffer salts. The grid was blotted after each drop to remove excess water and then stained with 10 μl of 1 % uranyl acetate adding a drop at a time and allowed to stand for about 5-10 sec. Excess stain was also removed by blotting with Whatman paper leaving a thin film on the surface of the grid. The grid was allowed to air dry for 3-5 min and stored in a Petri dish lined with filter paper for TEM examination. The grid was examined under a transmission electron microscope (JEOL 1220) with accelerating voltage of 80 kV. Digital images were captured using SIS Megaview III digital camera with analysis software.

2.6.2 Host range of induced phages

The host range of the phages induced was determined using plaque assays and spot tests on host strains of *C. difficile* (Mahony *et al.*, 1985, Cornax *et al.*, 1990, Sekulovic *et al.*, 2011).

2.6.2.1 Plaque assay

To carry out a plaque assay, bacteria hosts were grown in FAB medium to OD_{550} 0.7-0.9. BHI semi solid agar (with 0.4 % w/v agar) was prepared and cooled to about 50 ^{o}C. Approximately 3 ml aliquot of the BHI semi solid agar was dispensed into Bijou bottles. Freshly prepared phage suspension from mitomycin C and norfloxacin inductions were serially diluted in SM buffer. About 100 µl of the host bacteria suspension was added into the warm BHI semi solid agar followed by 200 µl of the diluted phage. This mixture was quickly mixed by gentle inversion and immediately poured onto pre-reduced BHI plates (without blood). Plates were allowed to set for 5 min at room temperature and then incubated at 37 ^{o}C in anaerobic chamber. Plates were checked after 24 h for plaque formation. Zones of lysis were then gently scrapped into SM buffer using sterile plastic loops or spatula and kept overnight at 4 ^{o}C. The mixture was then vortexed to mix followed by centrifugation at 2,100 $\times g$ for 10 min. The supernatant was aspirated and used to re-challenge the host in plaque assay as above.

2.6.2.2 Spot test

To carry out a spot test, BHI plates were prepared (without blood), labelled into quarters and pre-reduced. About 100 µl of BHI broth culture of a host strain at OD_{550} 0.7-0.9 was mixed with a 3 ml molten 0.4 % BHI semi solid agar and immediately poured onto the BHI agar plate. The plate was allowed to set at room temperature for 5 min thereafter 20 µl of phage suspension was applied on each segment and allowed to

dry for further 5 min. Plates were then incubated at 37 ^{o}C for 24 h in an anaerobic chamber.

Plaque assay and spot test were also carried out with salt (MgCl, MgSO$_4$, CaCl$_2$ and NaCl) supplemented in the semi solid agar at a final concentration of 0.5 M to enhance the activity of the phages as previously reported (Mahony et $al.$, 1985). To do this, double strength BHI semi solid agar (74 g/L BHI and 0.8 % w/v agar) and double strength salt solution (1 M of MgCl, MgSO$_4$, CaCl$_2$ and NaCl) were prepared and autoclaved. To prevent precipitation, the semi solid agar and the salt solutions were autoclaved separately. Just prior to plaque assay, 1.5 ml of warm double strength BHI semi solid agar was mixed with equal amount of the warm double strength salt solution and kept warm in a water bath (set at 55 ^{o}C). The hosts and the phages were added (as described in section 2.6.2) to the salt supplemented semi solid agar, mixed, poured unto pre-reduced BHI plates and incubated in at 37 ^{o}C in the anaerobic chamber for 24 h. For the spot test, the host was added to the salt supplemented semi-solid agar and poured unto labelled BHI plates. After cooling for about 5 min, 20 µl of the phage suspensions were applied on each section. The plates were allowed to dry for about 5 min before being incubated anaerobically for 24 h.

2.6.3   Pulsed-field gel electrophoresis (PFGE) of phage particles

PFGE was used to determine the whole genome size of the phages (Goh et $al.$, 2007). As PFGE requires a high DNA concentration, large scale prophage inductions were carried out and phage suspensions were purified to a very small volume using PEG in order to obtain high titre of phages. Therefore, 1 L each of bacterial cultures was prepared, induced and purified as described in sections 2.5.1 and 2.5.2 and a final volume of 2.5 ml of the samples were obtained after purification. The PEG purified lysates were then used for plugs formation.

2.6.3.1 Plug formation

The PFGE plugs were prepared as previously described (Wakita *et al.*, 2002). Briefly, the plug casting mould was sealed at the bottom using a piece of masking tape and the wells were labelled. Seaplaque CTG agarose (2 % w/v) (Cambrex Bio Science Wokingham, Berkshire, UK) in $0.5 \times$ Tris-Borate-EDTA (TBE, pH 8) was prepared and held warm in a boiling water bath. About 40 µl each of PEG purified phage samples was transferred into labelled eppendorf tubes followed by the addition of 60 µl warm plug agarose, which was gently mixed by gentle pipetting up and down and ensuring bubbles were not formed. Sterilised SM buffer was used as a negative control and a DNA sample of S-PM2, a cyanophage was used a positive control (the genome size of this phage was already known). The mixture was immediately transferred into the labelled gel moulds and allowed to set for 30 min at room temperature followed by a further incubation for 30 min at 4 ^{o}C. About 1 ml of lysis buffer with 0.5 mg/ml final concentration of proteinase K (proteinase K was added just before use) was added to a clean labelled eppendorf tube. The plugs were taken out of the 4 ^{o}C fridge and masking tape removed. The plugs were added to the lysis buffer by pushing them from the back of the mould and incubated in a water bath at 55 ^{o}C for overnight. The next day, agarose plugs were removed from the water bath and washed three times with $1 \times$ Tris-EDTA buffer (TE, pH 8).

2.6.3.2 Pulsed-field gel electrophoresis procedure

Approximately 200 ml of 1 % (w/v) Pulsed-field certified agarose (BioRad Laboratories, Carlifornia, USA) was prepared in 0.5 x TBE and brought to boil in a microwave. The PFGE tank and combs were assembled. The warm agarose was poured into the tank and allowed to cool at room temperature for 1 h. The gel was wrapped in a cling film and left at 4 ^{o}C for over night. The washed plugs were gently removed from

TE buffer and loaded into wells of the Pulsed field certified agarose gel using 70 % ethanol-sterilised spatulas. Using sterilised blade, 2 mm of the low range PFG marker (New England Biolabs, Herts, UK) was cut and loaded in one of the empty wells of the gel. The wells were sealed up with the remaining molten agarose. The products were separated in 0.5 × TBE buffer using a Bio-Rad CHEF-DR-II Pulsed Field Electrophoresis System (Bio-Rad, Richmond, CA) at 6 volt cm^{-1} for 15 h with a pulse ramp from 5-13 s at 14 °C. Afterwards, the gel was removed and stained with GelRed (Cambridge Biosciences, Cambridge, UK). The gel was visualised using Synegene box and images saved as computer files using Genesnap software.

2.6.4   Restriction fragment length polymorphism (RFLP) analysis of phage DNA

2.6.4.1 Phage DNA extraction from Qiagen midi kit

Phage DNA was prepared from induced culture suspension using Qiagen Lambda Midi kit (Qiagen Ltd, West Sussex, UK). Please refer to protocol in the kit.

2.6.4.2 RFLP procedure

Phage DNAs isolated using Qiagen midi kit (Section 2.6.4.1) were analysed by restriction fragment length polymorphism. Before the phage DNA was digested, it was mixed with 2 % DNA loading dye and ran through a gel to ascertain the presence of a reliable phage DNA weight. Suitable phage DNA samples were then selected and used for digestion using various enzymes. A total of ten restriction enzymes were used. These included seven 6-base pair enzymes (BamH1, Nde1, BstB1, HindIII, EcoR1, Sty1 and Sma1) and three 4-base pair enzymes (Sau3A1, Mbo1 and Dpn1). The enzymes Sau3A1, Mbo1, Dpn1 and BstB1 are known to cut methylated DNA (Zhu and Henney Jr, 1990, Rosado *et al.*, 2001, Pires *et al.*, 2004). The enzymes were used according to the manufacturers' instructions. Digestion was performed overnight.

Where necessary the digestion mixture was heated at 65 °C for 20 min to inactivate the enzymes and to prevent any cohesive ends joining. The digestion mixture was then separated through   1 % Helena Agarose gel prepared in 1×TAE containing 0.1 μl/ml GelRed. The gel was run in 1 x TAE buffer for 1 h 30 min (150 mA).

2.7   Molecular makers for *C. difficile* phages

Three degerate sets of PCR primers were designed to determine the molecular diversity of the phages associated with my strains. The PCR primers targeted three *C. difficile* myovirus genes which include: the capsid, holin and portal genes. The three genes appeared to be conserved in part and variable in part when five *C. difficile* myoviruses were analysed (Goh *et al.*, 2007).

2.7.1   Isolation of phage DNA by Phenol/chloroform/isopropanol method

The phage DNA was extracted using phenol/chloroform/isopropanol method (Bouillaut *et al.*, 2005, Fortier and Moineau, 2007, Nale *et al.*, 2012). About 0.1 μl of DNase 1 and RNase A (Promega BioSciences, CA, USA) were added to 700 μl of PEG purified phage suspension. The mixture was incubated for 1 h at 37 °C followed by the addition of equal volume of phenol and incubated at room temperature for 2 min before being centrifuged at 15,000 × *g* for 10 min. The aqueous layer was then extracted with an equal volume of phenol/chloroform/isopropanol (25:24:1, v/v/v) solution, mixed well and left for 2 min at room temperature before being centrifuged at 15,000 × *g* for 10 min. (The phenol/chloroform/isoamyl solution was prepared at least 24 h before usage to allow the solution to settle. The solution is collected from the bottom layer). The aqueous layer was extracted by mixing with an equal volume of chloroform/isopropanol (24:1 v/v), incubated for 2 min at room temperature and then centrifuged at 15,000 × *g* for another 10 min. The resulting aqueous layer was then

mixed with 0.4 volumes of 7.5 M ammonium acetate and 2 volumes of isopropanol and incubated on ice for 1 h before being centrifuged at $21,000 \times g$ for 20 min at 4 ^{o}C. The supernatant was carefully removed and the resulting DNA pellet was washed once with 500 µl of 70 % alcohol followed by a final centrifugation of 20 min at $15,000 \times g$ to recover the DNA pellet. The DNA pellet was briefly air-dried for 2-3 min and then dissolved in 30 ml 10 mM Tris-Cl elution buffer. A NanoDrop ND-1000 spectrophotometer was used to quantify the phage DNA concentration in the samples.

2.7.2   Primer design

2.7.2.1 Capsid and portal genes

The capsid and portal genes primers were designed by aligning four known *C. difficile* phage capsid gene sequences. The four sequences were the only *C. difficile* phage sequences available at the time of this study. Three of the four sequences CD119, phiC2 and CD630 were obtained from NCBI searches and the fourth (phi12) was a partial DNA sequence of a *C. difficile* temperate myovirus sequenced in our laboratory by Katherine Hargreaves. A BLAST search for each of these putative primers was performed to determine the specificity of the genes. The sequences were aligned using ClustalW 2.0.12 multiple sequence alignment tool and conserved regions, for the forward and reverse primers were manually selected. The sequences for the reverse primers were generated using the online Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html). Differences within the sequences were accounted for by using the International Union of Pure and Applied Chemistry (IUPAC) nucleotide ambiguity codes. The stability of the primers sets were determined by calculating their GC contents and a minimum GC content of 40 % was allowed. The

primers were then acquired and 100 µM stock solutions of the primers were produced and stored in -20 ^{o}C for future use.

2.7.2.2 Holin gene

The set of degenerate PCR primers targeting the *C. difficile* phage holin gene were designed by Dr Jinyu Shan in or laboratory. The primers were designed from the alignment of three *C. difficile* phages (phiCD119, phiCD27, and phiC2), and 4 *C. difficile* strains (CD630, R20291, CD196, and QCD-63q42) (Shan *et al.*, 2012).

Before the primers were used for PCR amplification, the specificity of each of the of primer set for the three genes were imputed into the online *in silico* PCR amplification (http://insilico.ehu.es/PCR/) and ran against all available *Clostridium spp* sequences with no mismatches allowed. The correct PCR product sizes were verified. The sequences for the PCR products were downloaded and verified that they were the correct genes using BLAST. Having ascertained the specificity of the primers, they were then used to amplify DNA of *C. difficile* phages and their genomic DNA.

2.7.3 PCR optimisation and amplification

The PCR conditions for each of the primer sets were optimised by adjusting the annealing temperatures and the final concentrations of the $MgCl_2$ used. Each set of primers were used to amplify DNA from 15 selected *C. difficile* 027 temperate phages including 10 myoviruses and 5 defective myoviruses. The phages were selected based on their abilities to be induced and abundant as confirmed by the TEM analysis. The primers were also optimesed by performing PCR amplifications on the host genomic DNA of the *C. difficile* 027 strains and nine additional isolates belonging to different ribotypes. A set of four controls was used. The first was a negative control in which no DNA template was used (an equivalent of sterile water was used to replace the volume

of DNA to be used). In the second control, a DNA template of S-PM2, a cyanophage was used. The third control had the DNA of phi12. As mentioned above, phi12 was a purified *C. difficile* temperate myovirus and the partial DNA sequence was used in the primer design of the capsid and the portal genes. Finally, the fourth control had the DNA of phiCD27, a purified *C. difficile* myovirus.

2.7.3.1 The PCR amplification conditions

Separate PCRs for the capsid and holin genes primers were carried out in a total volume of 25 µl, containing 4 µM forward and reverse primers, 0.25 mM dNTPs, 3mM MgCl$_2$, 2.5 µl of 10 × PCR reaction buffer, ~50 ng of template DNA, 0.2 unit of Taq polymerase (Bioline, London, UK). Amplification conditions were: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 sec, annealing at 48°C for 45 sec, extension at 72°C for 1 min, with a final extension of 10 min at 72°C.

2.7.3.2 Portal gene amplification condition

For the portal gene primer, a total volume of 25 µl was set for the PCR reaction, and contains 4 µM forward and reverse primers, 0.25 mM dNTPs, 4mM MgCl$_2$, 2.5 µl of 10 × PCR reaction buffer, ~50 ng of template DNA, 0.2 U of Taq polymerase. Amplification conditions were: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, extension at 72°C for 1 min, with a final extension of 10 min at 72°C.

The PCR amplification was carried out in a LabCycler (SensoQuest GmbH, Göttingen, Germany). PCR products were resolved in 1 % Agarose (Helena Biosciences, Tyne and Wear, UK) prepared in 1×Tris-acetate-EDTA (TAE, pH 8) containing 0.1 µl/ml GelRed at 100 V for 1 h 30 min. The gels were removed and visualised using Synegene box and images saved as computer files using Genesnap software.

2.7.4 Cloning of the amplified genes

The amplified phage gene products were gel purified and cloned using TOPO TA cloning. In this method, a linearized plasmid vector (pCR®2.1-TOPO®) with a single 3´ thymidine (T) overhang and a topoimerase 1 covalently bound to it was used. The insert (the PCR amplified gene product) has a single deoxyadenosine (A) added to its 3´ ends by the *Taq* polymerase. This allowed PCR inserts to ligate efficiently with the vector.

2.7.4.1 Isolation of phage DNA from agarose gel

DNA from the PCR products was extracted and purified from the gel using NucleoSpin® Extract II (Fisher Scientific, Leicestershire, UK). The DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer. The purified PCR products were used within 8 h of purification for cloning, or stored at 4 ^{o}C for a maximum of 48 h. After 48 h of storage, the inserts may lose their A over-hang and were incubated with *Taq* polymerase at 20 ^{o}C for 1 h to add the A over-hang before being used for cloning.

2.7.4.2 Production of *E. coli* competent cells

E. coli (DH5α-T1) cells were cultured on LB plate at 37 ^{o}C for 18-24 h. A single colony was used to inoculate 5 ml of Luria-Bertani (LB) broth and incubated over night. About 2 ml of the overnight broth culture of the *E. coli* was used to inoculate a fresh 200 ml LB broth and incubated at 37 ^{o}C while shaking at 200 rpm. At OD_{600} of 0.35 (after about 1 h), the culture was heat shocked by placing on ice for 20 min followed by aliquoting 40 ml into ice-cold centrifuge tubes (subsequent treatment of the cells was done on ice and all tubes and pipette tips were refrigerated at 4 ^{o}C prior to use). The aliquots were immediately centrifuged at 3,700 × g at 4 ^{o}C for 10 min. The resulting pellets were resuspended in 30 ml ice-cold 100 mM $CaCl_2$. Pellets were recovered again by centrifuging as above. Finally, the pellets were resuspended in 8 ml

of ice-cold 100 mM CaCl$_2$ containing 15 % glycerol and 80 ul aliquots were made and immediately stored at -80 for future use.

2.7.4.3 Production of T-vectors

Due to the fact that a large number of samples were examined in this study, it was cost effective to propagate my own vector. To do this, a single blue colony from a previous TOPO-TA cloning was propagated by streaking on a LB medium supplemented with 100 mg/ml Ampicillin or Kanamycin and spread with 40 μl X-gal (40 mg/ml). The blue colonies did not have the inserts but possessed the resistance genes for Kanamycin and Ampicillin. After 24 h incubation, a single colony was inoculated into 10 ml of LB broth supplemented with 100 mg/ml. Ampicillin or Kanamycin (Sigma-Aldrich, Dorset, UK) and incubated for 18 h. The cells were harvested by centrifugation at 15,000 × g for 5 min. The resulting pellet was used for plasmid isolation using GeneElute plasmid mini preparation kit (Sigma-Aldrich, Dorset, UK). The plasmid was then eluted in 5 mM Tris-Cl buffer, pH 8.0 digested with a blunt-end cutter, EcoRV (New England Biolabs, Herts, UK). Afterwards, the mixture was heated at 70 ^{o}C for 10 min to stop the activity of the enzyme. The blunt-end vector was purified using PCR purification kit (Qiagen, West Sussex, UK) and its quality determined using a NanoDrop ND-1000 spectrophotometer. A 10 μl volume of T-tailing reaction was set up containing ~5 μg of blunt-end vector, 1 x PCR buffer, 0.1 U *Taq* polymerase and 2 mM dTTP. The reaction was incubated at 72 ^{o}C for 2 h and afterwards separated through a PCR column (Qiagen, West Sussex, UK) by centrifuging at 15,000 × g for 10 min. The quality of the vector was checked again using a NanoDrop ND-1000 spectrophotometer. The purified vector was then used immediately in ligation with a freshly prepared gel purified PCR insert as described in section 2.7.4.1.

2.7.4.4 Setting up TA cloning

A 10 µl volume TA cloning reaction was set up in a 100 µl volume PCR tube. The reaction contained 2 ng of PCR insert, ~50 ng freshly prepared T-vector, 2 µl of 5 x Ligase buffer and 0.3 U of T4 ligase (Bioline, London UK). The ligation reaction was incubated at room temperature for 12-18 h without shaking. LB plates supplemented with 100 µg/ml of Ampicillin/Kanamycin were incubated at 37 °C overnight to warm the plates. The next day, 40 µl of X-gal was spread on the antibiotic selective LB plates and further warmed at 37 °C for about 1 h. A water bath was equilibrated to 42 °C and one vial (80 µl) of *E. coli* DH5α-T1 competent cells was thawed on ice. About 5 µl of the T-A cloning reaction mixture was gently added to the competent cells. This was then mixed gently by tapping the bottom of the tubes (do not mix by pipetting) and incubated on ice for 1 h. The transformed competent cells were then heat-shocked for 30 sec at 42 °C water bath without shaking before being quickly transferred unto ice. About 150 µl of SOC medium (at room temperature) was added and the tubes were shaken horizontally (200 rpm) at 37 °C for 1 h. Afterwards, about 80 ml of the transformed chemically competent *E. coli* cells were spread onto pre-warmed antibiotic selective LB medium with X-gal at 37°C. Plates were observed after 18-24 hr of incubation. Four randomly selected white colonies suspected to contain the insert were picked and re-streaked onto new antibiotic selective LB plates spread with X-gal and cultured aerobically for 18-24 h.

2.7.5 Plasmid preparation

A single white colony from the overnight selective plate was inoculated into 10 ml LB broth containing 100 µg/ml of Ampicillin/Kanamycin and incubated aerobically at 37 °C for 18-24 h. Cells were spun down and plasmid extraction was performed using Sigma GeneElute plasmid mini preparation kit. Final plasmid products were

washed with 25 µl of elution buffer. Plasmids were digested with EcoR1-HF (New England Biolabs, Herts, UK) to confirm the inserts. The enzyme digestion system was carried out in a total of 10 µl volume containing 1 U of the enzyme, 800-1000 ng/µl of plasmid DNA and 1 x PCR buffer. The reaction mixture was incubated at 37°C overnight. Afterwards, the reaction mixture was heated at 62 ^{o}C to inactivate the enzyme. The resulting products were mixed with 2 µl of 5 x DNA loading buffer (Bioline, London UK) and resolved in 1 % Helena Agarose gel prepared in 1 × TAE containing 0.1 µl /ml GelRed. The gel was run in 1 × TAE buffer at 90V for 1 h 30 min (150 mA). Positive plasmids containing the inserts were selected for sequencing. Sequencing was carried out by GATC Biotech using M13 forward (5´-GTAAAACGACGGCCAG-3´) and reverse (5´-CAGGAAACAGCTATGAC-3´) primers.

2.7.6 Analysis of the cloned PCR products

The chromatogram files (.abi format) of the plasmids sequences were retrieved from GATC website and edited using Chromas 2.33. All the sequences were truncated by 20 nucleotides downstream and upstream to eliminate any miss-appropriations on the insertion points in the chromatogram files. The fasta format of the sequences was generated and the sequences were analysed using nucleotide BLAST (blastn, NCBI) to confirm the gene identity. The sequences were then translated into amino acids using Transeq sequence analysis in EMBL-EBI-tool-sequence analysis-Transeq (Algorithm used was Frame-6, Table-Standard Code, Regions-Start-end, Trim-No, Reverse-No and Colour-No). One of the frames with no or minimum stop codons (indicated by an asterix sign) was chosen for further analysis. The translated proteins were again confirmed using protein BLAST (blastp, NCBI). Alignment of the protein sequences was done using ClustalX2. The files (.phy) generated from ClustalX2 were imported

into MEGA5 software and analysed. A new alignment was built in MEGA5 and the Neighbour-Joining tree was generated using *p*-distance method with boostrap replication of 1000. Branches were proportionate to the length and strains with a boostrap proportion of at least 75 were assigned into a clade.

2.8 Induction, isolation and purification of *C. difficile* spores

2.8.1 Spore induction on solid medium

C. difficile 027 spores were produced using the basic protocol 4 (Sorg and Dineen, 2009) with slight modifications. About 10 % of overnight liquid culture of *C. difficile* in fastidious anaerobe broth was transferred into freshly pre-reduced BHI broth. Bacteria cultures were incubated at 37 ^{o}C anaerobically until an OD_{550} was ~0.7-0.9 as achieved. BHI agar (without blood) was prepared and about 5 ml was dispensed into 6-well culture plates, allowed to set and pre-reduced for 1 h. Approximately 150 µl of bacterial culture was transferred to the wells of the prepared microtiter plates containing BHI agar. One plate was used for each sample. The plates were then incubated anaerobically at 37 ^{o}C for 7 days to induce sporulation. Afterwards, the bacterial culture from the top of the wells were gently scraped off using sterile inoculation loops and placed into a 15 ml capacity tube containing 1 ml of ice cold-water. Residual vegetative cells and spores were recovered by washing the plate wells with ice cold water to obtain a total of 10 ml spore suspension in the tube for each sample. About 4 µl of the spore suspension was placed on a slide, covered with a cover slip and the spores were inspected using a microscope. The spore/vegetative cells suspension was then centrifuged for 1 min at 14,000 × *g* at room temperature. Supernatant was gently removed and the washing and centrifugation steps were repeated 9 additional times. Afterwards, 20 % and 50 % (w/v) HistoDenz solutions (Sigma-Aldrich, Dorset, UK) were prepared in ultra pure water. Final pellet was then re-suspended with 20 % w/v

HistoDenz and combined to make a total of 5 ml. Using a 5 ml capacity Pasteur pipette, this suspension was gently lowered onto 10 ml 50 % HistoDenz in a 15 ml tube (Care was taken not to disturb the density interphase) (Paredes-Sabja *et al.*, 2009, Howerton *et al.*, 2011, Paredes-Sabja and Sarker, 2011). This was then centrifuged for 20 min at 15,000 × *g* and 4 ^{o}C. The supernatant was then gently removed (debris at the interphase was removed first) and the pellet at the bottom of the tube containing the spores was re-suspended in 1 ml ice cold water. The spores were washed once with ice cold water and finally resuspended in 200 µl of ice cold water. To completely eliminate vegetative cells, the spore suspension was heated at 60 ^{o}C for 20 min on a heating block. The concentration of spores in the preparations was then quantified by determining the viable count. To do this, spore samples were serially diluted in ultra pure water. BHI agar plates containing 0.1 % (w/v) each of L-cysteine (Sigma-Aldrich, Dorset, UK) and Sodium taurocholate (Sigma-Aldrich, Dorset, UK) were prepared (Buggy *et al.*, 1983, Buggy *et al.*, 1985). The plates were pre-reduced anaerobically at 37 ^{o}C for 1 h prior to use. A 20 µl volume of the diluted spore preparations were applied unto plates and spread evenly using a sterile plastic spreader. The plates were then incubated anaerobically for 24 h at 37 ^{o}C to allow the spores to germinate and grow. Two sets of negative controls were included. One was the plating of 20 µl of ice cold distilled water on BHI plates supplemented with L-cysteine and Sodium taurocholate salt. The second control was the plating of 20 µl of the prepared spore suspension on plain BHI with no supplements. After 24 hr incubation, number of colony per plates were counted and the CFU/ml count was done using the formula below

$$CFU/ml = \frac{\text{Number of colonies formed}}{\text{Volume plated (ml)}} \times \text{dilution factor}$$

2.8.2 Spore induction in liquid medium

Approximately 2 ml of *C. difficile* overnight culture in FAB was added to 7 ml of pre-reduced BHI broth and grown anaerobically at 37 ^{o}C whilst being shaken at 80 rpm for 168 h. Spore initiation was monitored daily by Gram-staining 10 µl of the bacterial culture on a slide and examined under phase-contrast microscope. After the 168 h, the samples were removed from the anaerobic chamber and placed in 4ºC fridge for a further overnight incubation. Afterwards, equal amount of 95 % v/v ethanol was added to the cultures and vortexed for 30 sec to mix. This mixture was incubated for 45 mins at room temperature before being centrifuged at 15,000 $\times$ *g* for 20 min. The supernatant was removed and discarded and pellet was resuspended with 5 ml of 20 % w/v HistoDenz. The pellet mixture was then gently lowered onto a 10 ml 50 % w/v HistoDenz in a 15 ml tube and centrifuged for 20 min at 15,000 $\times$ *g* and 4 ^{o}C. The spores were purified and titred as described in section 2.8.1 above.

2.8.3 Preservation of spores

The spores were cryo-preserved in Protect Bacterial Preservers. To do this, about 100 µl of glycerol was recovered from the Protect Bacterial Preservers vials and disposed of. This was then replaced with equal amount of the prepared spores, mixed by inversion and immediately stored at -80 ^{o}C.

2.8.4 Scanning electron microscopy of spores (SEM)

The spores were treated with 2 % glutaraldehyde, 37-40 % formaldehyde and 1000 ppm (parts per million) presept to inactivate them. The spore inactivation was done from 0 min to 3 h. The CFU/ml counts of the treated spore samples were taken at 30 min time interval within the 3 h inactivation period. At the end of the 3 h treatment, the morphology of the inactivated spores was eaxmined using SEM analysis performed

at Electron Microscopy Laboratory, Core Biotechnology Services, College of Medicine, Biological Sciences and Psychology, Adrian Building, University of Leicester, UK.

2.8.5 Data Analysis of sporulation characteristics of *C. difficile* isolates

All experiments were performed in duplicate or triplicate on at least three separate occasions. Comparative spore counts from the various strains were analysed using a One-way ANOVA (Analysis of Variance) in GraphPad Prism 5 (Appendix 10). Statistical significance was indicated by a P value of 0.05.

3 Isolation and characterisation of temperate bacteriophages of *Clostridium difficile* 027 strains

3.1 Introduction

Temperate bacteriophages, otherwise known as lysogenic bacteriophages are phages that exist in lysogenised form and can remain in a dormant inactive form in their hosts. In the latent form, a prophage generally integrates its genome into the chromosome of the bacterial host (the lysogen) and continues to replicate along with the bacteria in a process called lysogeny. Alternatively, it can exist as a plasmid, independent of the host's chromosome but segregate at regular fashion during cell division (Cann, 1993). However, these phages are capable of switching to a lytic pathway either spontaneously or through induction with uv rays, alkylating agents (such as mitomycin C), fluoroquinolones (such as norfloxacin) or ionising radiations (Driggers and Schmidt, 1970, Lydersen and Pollard, 1975, Goldstein, 1987, Matsushiro *et al.*, 1999).

During lysogeny, genes that are not necessary for their existence may be transferred to the bacterial host through horizontal gene transfer resulting in the conversion of the host phenotype (James *et al.*, 2001, Saunder *et al.*, 2001, Bruggemann, 2005).

Although lysogenic conversion has not been reported in *C. difficile*, a significant relationship between phages and their lysogenic hosts has been reported (Govind *et al.*, 2009). It was reported that phage infection of toxigenic *C. difficile* strains has resulted to increased production of ToxA and ToxB in a lysogen by two and four folds respectively (Goh *et al.*, 2005a, Govind *et al.*, 2009). In addition, an amino acid

homologous to *tcd*E in the PaLoc of *C. difficile* was identified in the genome of three *C. difficile* phages (Goh *et al.*, 2005a). Recently, a temperate siphovirus from *C. difficile* was characterised. Infection of *C. difficile* strains with the siphovirus led to increase in toxin production in the hosts (Sekulovic *et al.*, 2011). Similarly, studies on *C. perfringens* also suggest the occurrence of interaction between phages and sporulation (Zimmer *et al.*, 2002).

Recent genomic analysis has identified mobile genetic elements including prophages in 22 of 25 sequenced *C. difficile* 027 strains, but the bacteriophage content was not characterised further (Henn *et al.*, 2010). In another study, when a *C. difficile* 027 outbreak strain was investigated, only single bacteriophage morphology was observed (Fortier and Moineau, 2007). Thus, information on *C. difficile* 027 phage carriage is grossly insufficient. In addition, no previous studies have examined bacteriophage carriage within *C. difficile* ribotype 027 subgroups. *C. difficile* 027 is widespread with various disease severities which complicate epidemiological studies (Fawley *et al.*, 2008, Morgan *et al.*, 2008). To determine if bacteriophages are contributing to the diversity within *C. difficile* 027, the range of temperate bacteriophage types associated with ribotype subgroups must first be established.

3.2   Aims and objectives of study

1. To induce prophages from clinical *C. difficile* 027 subtype isolates using norloxacin or mitomycin C

2. To characterise phages based on their

    a. morphology using transmission electron microscopy

    b. genome size using pulsed-field gel electrophoresis

    c. host range using plaque assays and spot tests

d. enzyme restriction profiles using restriction fragment length polymorphism (RFLP) analysis

3. To determine if phage carriage correlates to subtype and disease severity

4. To compare the efficacy of norfloxacin and mitomycin C to prophage induction in *C. difficile*.

3.3 Results

3.3.1 Morphological diversity of temperate bacteriophages of *Clostridium difficile* 027 strains

This study was designed to induce and characterise temperate bacteriophages of *C. difficile* 027 as a step to understand their potential role in disease and diversity of this pathogen. The first step is to establish if all *C. difficile* 027 strains contain phages with the same morphology as previously proposed (Fortier and Moineau, 2007). My previous finding (MRes research) suggested that this was not the case and that diverse phage morphology exists in the genomes of *C. difficile* 027 strains isolated from University Hospital of Leicester (Nale, 2009, Tromans *et al.*, 2010). Again, my previous work had shown that many of the *C. difficile* strains examined could not be induced with mitomycin C. To extend on this data, within the context of well characterised strains, a set of 91 *C. difficle* 027 strains were examined. All the 91 *C. diffcile* 027 strains were induced with norfloxacin or mitomycin C at a final concentration of 3 μg/ml of either antibiotic. TEM analysis following induction and purification of lysates using PEG revealed a diverse set of phage morphologies associated with *C. difficile* ribotype 027 (Nale *et al.*, 2012). Ninety of the induced 91 strains yielded morphologies of tailed phages belonging to the *Myoviridae*, the *Siphoviridae* or the phage tail-like particles (Figure 3.1, Figure 3.2, Figure 3.3). A detailed table describing all of the phages induced from the individual isolates is given in Appendix 2. The table shows the different MLVA and Pulsovar subtypes of the isolates and the phage TEM images induced from them.

3.3.1.1 Myoviruses

Intact myoviruses (morphology A) were only induced from two isolates and all had hexagonal capsids of ~70 nm in diameter which were attached to the tail by a short

75

portal protein (Appendix 2, Figure 3.1) (The phage dimension was estimated by taking a mean measurement of six phages in each sample). The contractile tails were ~200 nm by 20 nm with horizontal striations on the sheaths which end in 5-6 ~50 nm long tail fibres. The two isolates yielding these intact myoviruses also yielded defective myoviruses (Morphologies B, C and D) (Figure 3.1, Figure 3.4). The defective myoviruses were the only viral particle morphology induced from a further 59 isolates (Appendix 2, Figure 3.1, Figure 3.4). These typically had less dense capsids and contracted tail sheaths with the wider part of the sheath at various positions along the remaining tail tube (Morphologies B, C and D) (Appendix 2, Figure 3.1). Some of the defective myoviruses had very long tail fibres such as those of morphology D (Figure 3.1). One additional isolate that yielded a defective myovirus also yielded a siphovirus (isolate 66L) (Appendix 2, Figure 3.4). A novel *C. difficile* myovirus (morphology E) was induced from one isolate. This viral particle had a small hexagonal capsid of ~40 nm diameter and tail of ~ 20 nm in diameter and 220 nm long (Appendix 2, Figure 3.1). The capsid was joined to its tail by a thin portal protein-like structure of ~10 nm in length and width. The tubular tail had an average of 55 striations $\pm$ 3 (based on an average of six) and the sheath ended with around 6-7 tail fibres (Figure 3.1).
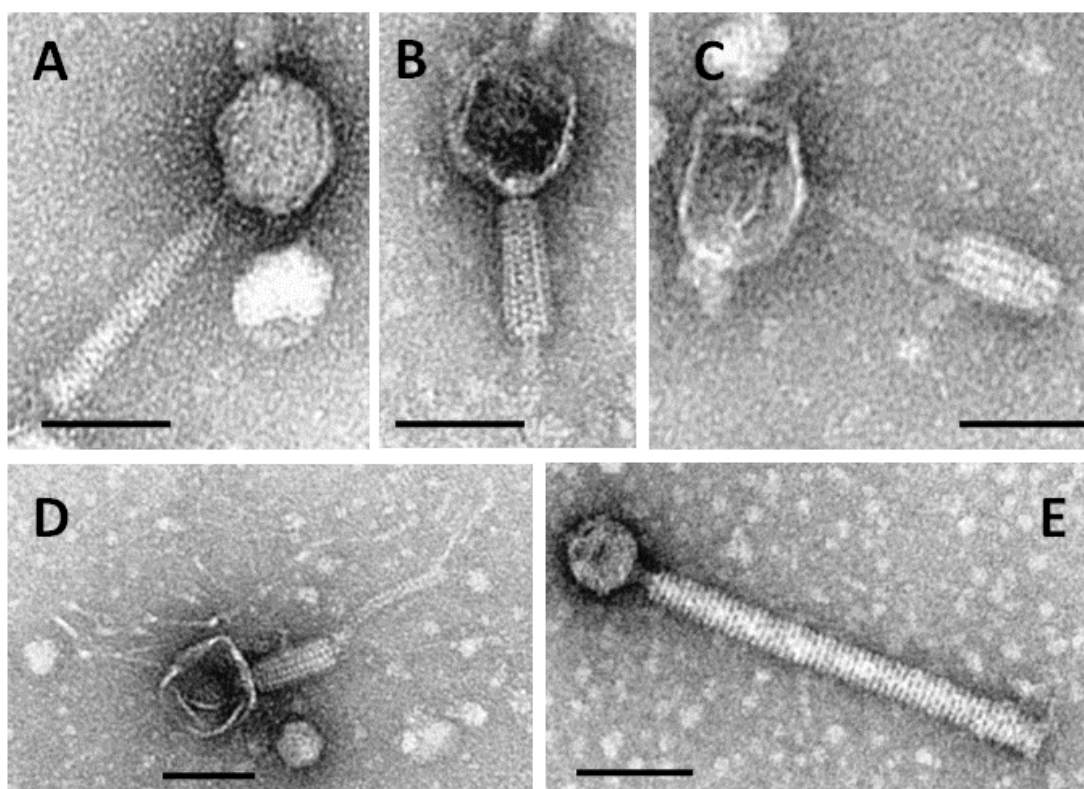
Figure 3.1 Diverse morphologies of myoviruses induced in this study.

C. difficile 027 isolates were induced using mitomycin C or norfloxacin at a final concentration of 3 μg/ml. All isolates were induced at least 4-6 occasions. Prophages from the filtered induced lysates were analysed using TEM. Typical myoviruses (morphology A) were induced from two isolates (96L and 91L) along with defective myoviruses morphologies B (induced from 96L) and C (induced from 91L). An additional morphology of defective myovirus, morphology D was induced from 20L. Fifty-nine isolates yielded defective myoviruses as the only viral particles. One strain that yielded a defective myovirus also yielded a siphovirus (isolate 66L). Myovirus with morphology E was only induced from one isolates (16L). Bars represent ~70 nm. Measurement was estimated by measuring 6 phages in each sample.

3.3.1.2 Siphoviruses

Intact siphoviruses typified by morphology F were released from three of the isolates (Appendix 2, Figure 3.2, Figure 3.4). These all had dense isometric capsid of ~70 nm in diameter with a long flexible non-contractile tail which is ~230-350 nm long and ~15 nm wide. Two morphotypes of siphoviruses were observed and both were of

type B1 with the capsid forming at least one third of the entire viral structure (Ackermann 2005) (Figure 3.2). The first siphovirus morphotype, Morphotype F1 had a capsid size of ~70 nm and tail length of ~350 nm. The tail of Morphotype F1 had about ~60 clear striations and ends in a base plate (Figure 3.2A). The second siphovirus morphotype, Morphotype F2 had a similar capsid size as that of Morphotype F1 but had two fibres beneath the capsid (Figure 3.2) (the fibres are indicated using white arrows). In addition, the tail of Morphotype F2 which is ~230 nm long with approximately 45 striations is considerably shorter than that of Morphotype F2 (Figure 3.2).
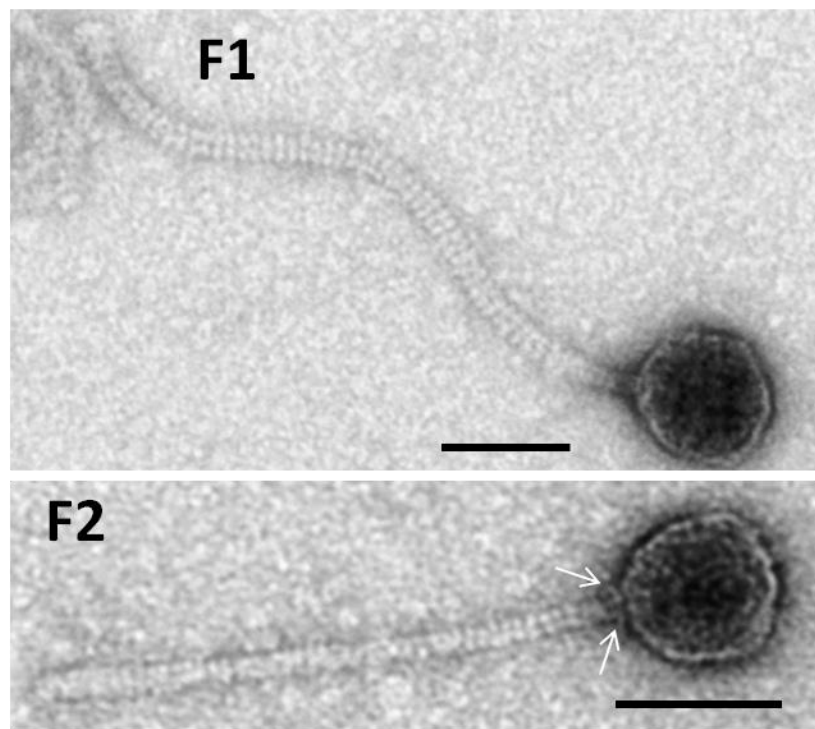
Figure 3.2 Siphoviruses induced from isolated examined in this study

Above shows the two morphotypes (F1 and F2) of siphoviruses induced from three isolates (48L, 37L and 66L) examined in this study. Bacterial cultures were induced on at least 4-6 occasions using mitomycin C or norfloxacin at a final concentration of 3 µg/ml. Prophages from the filtered lysates were analysed using TEM. Morphotype F1 (induced from 48L) had a capsid of ~70 nm and tail of ~350 nm long and ~15 nm wide. Morphotype F2 (induced from 48L, 66L and 37L) had the same capsid size as Morphtype F1 but the tail is ~230 nm long and possesses fibers as indicated by white arrows. Bars represent ~70 nm. Measurement was estimated by measuring 6 phages in each sample.

3.3.1.3 Phage tail-like particles

Twenty-six isolates produced phage tail-like particles only (Appendix 2, Figure 3.3, Figure 3.4). There were two discrete categories of phage tail-like particles identified. One category had particles which looked like genuine phage tails and were ~160 nm by 20 nm terminating in horizontal protrusions (morphology G) (Figure 3.3). These phage tail-like particles were induced from all isolates (in addition to other morphologies) (Appendix 2). Two isolates harboured particles with morphology belonging to the second category (Morphology H) (Figure 3.3). The second particle type had tightly coiled spirals approximately 20 nm diameter and between 80-350 nm long. Only one isolate did not yield any prophage particles under the induction procedure discussed above.
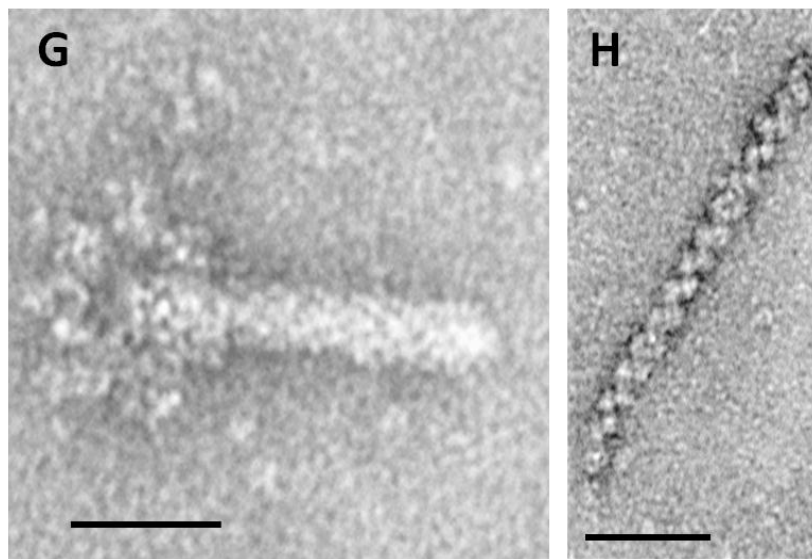

Figure 3.3. Phage tail-like particles induced from isolates examined this study.

The phage tail-like particles G and H above were induced from isolates 47L and 40L repectively. The phage tail-like particles with morphology G was induced from 23 isolates as the only viral particles. These phage tail-like particles were also induced from all the cultures along with other morphologies. The phage tail-like particles H was induced from 2 of the isolates as the only viral particles. Bars represent ~70 nm. Measurement was estimated by measuring 6 phages in each sample.

3.3.1.4 Dual phage carriage

Dual phage carriage was observed in four isolates; one isolate had a defective myovirus and a siphovirus (morphotype F2) (66L), two had a defective myovirus and an intact myovirus (96L and 91L) and finally one had two morphotypes of siphoviruses (48LM) (Appendix 2, Figure 3.4Figure 3.4). The two morphotypes of siphoviruses are shown in Figure 3.2 and have been discussed in section 3.3.1.2 above. Since phage tail-like particles were present in all the induced isolates that carry phage particles, this morphology was not considered in the dual phage carriage analysis.
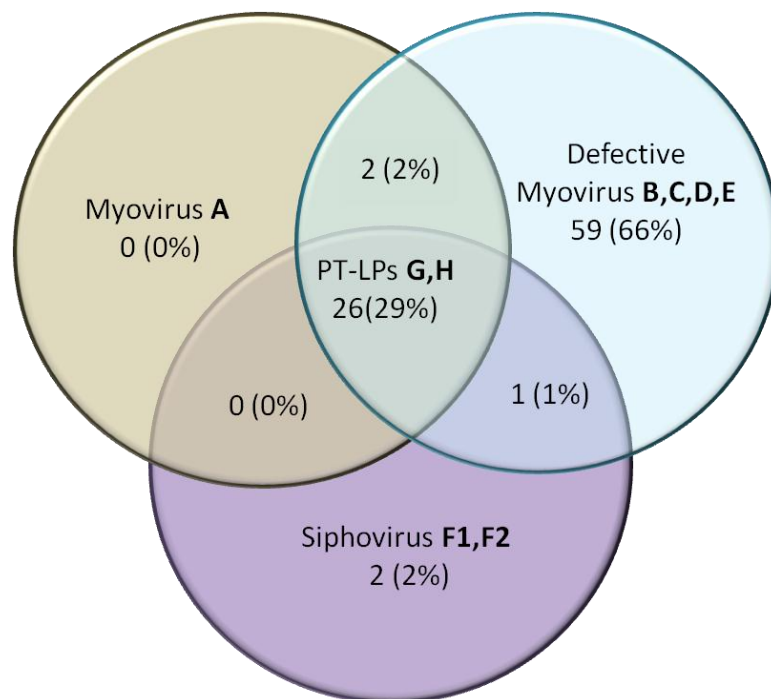
Figure 3.4 Proportion of phage morphologies induced from the 91 *C. difficile* 027 isolates used in this study.

Above shows the proportion of diverse bacteriophages induced from 90 out of the 91 of *C. difficile* 027 isolates examined in study. The diverse morphologies consisted of putative myoviruses (Myovirus A) induced from two isolates, defective myoviruses B, C, D and E induced from 60 isolates and two siphovirus morphotypes (F1 and F2) induced from three isolates. Phage tail-like particles (PT-LPs) were induced from 26 isolates as the only phage particles but were also induced from all the other isolates in addition to other morphologies. Dual phage carriage was observed in four isolates; two isolates yielded myovirus A and defective myoviruses B and C (isolate 91L and 96L), one isolate yielded a siphoviurs (F2) and defective myovirus (B) (isolate 66L) and one isolate (48L) yeiled two morphortypes (F1 and F2) of siphovirus.

3.3.2 Correlation of phage carriage to ribotype 027 subtypes

It was earlier mentioned that the 91 *C. difficile* 027 strains induced in this study have been categorised into different 23 MLVA and 5 Pulsovar types. To determine if the diverse temperate phage carriage observed in this set of samples contribute to diversity, the phage carriage was correlated to the MLVA and Pulsovar types. Interestingly, a correlation between phage carriage to the MLVA types and Pulsovar types was observed (Table 3.1).

MLVA types 1-15 are comprised of a total of 61 isolates and yielded only defective myoviruses apart from three isolates which consist of 66L, 96L and 91L in MLVA 12, 13 and 15 respectively. It was observed that in addition to the defective myoviruses these isolates also contained a typical myovirus (96L, 91L) or a siphovirus (66L). MLVA types 16 (15 isolates), 17 (5 isolates), 19 (1 isolate), 20 (2 isolates), 21 (1 isolate), 22 (2 isolates) and 23 (1 isolates) were found only to contain phage tail-like particles with one exception each in MLVA 16 (isolate 16L which also yielded a novel myovirus E) and MLVA 22 (isolate 36L which did not yield any phage particles under the induction conditions above). There were three isolates classified as MLVA 18 and two had inducible siphoviruses and one, a defective myovirus (Table 3.1, Appendix 2).

Pulsovar I (containing 14 different MLVA types and a total of 53 isolates) was strongly associated with the production of defective myoviruses. All the induced isolates contained defective myoviruses except isolate 71L which contained only phage tail-like particles. Pulsovar II was only represented by one isolate and contained defective myoviruses. Two isolates from Pulsovar III were examined and both contained siphoviruses. It could be recalled that Pulsovars II and III formed MLVA 18. The one isolate that yielded defective myovirus in MLVA 18 as indicated above belongs to the Pulsovar II and the other two that yielded siphoviruses are in Pulsovar

III. Therefore phage carriage is more Pulsovar sensitive in this case compared to the MLVA type. Thirty-three isolates from Pulsovar IV were examined and they all yielded phage tail-like particles. In addition, ten isolates also yielded defective myoviruses. Pulsovar V consists of two isolates; one contained phage tail-like particles and the other (36L) yielded no phage (Table 3.2, Appendix 2).

The majority of the MLVA types 17/23 (~74 %) had specific phage carriage with no exceptions while 2/5 (40 %) of the pulovar types contained specific phages. Therefore, generally, stronger correlation of phage carriage to the MLVA types compared to the Pulsovar types was observed.

Table 3.1 Phage carriage in 91 *C. difficile* 027 isolates in relation to their MLVA types.

MLVA type	Number of isolates	Morphology of phage induced	Exceptions
1	3	Defective myovirus	-
2	1	Defective myovirus	-
3	5	Defective myovirus	-
4	7	Defective myovirus	-
5	1	Defective myovirus	-
6	2	Defective myovirus	-
7	7	Defective myovirus	-
8	2	Defective myovirus	-
9	2	Defective myovirus	-
10	1	Defective myovirus	-
11	3	Defective myovirus	-
12	6	Defective myovirus	66L (Siphovirus F)
13	12	Defective myovirus	96L (Myovirus A)
14	3	Defective myovirus	-
15	6	Defective myovirus	91L (Myovirus A)
16	15	PT- LPs*	16L (Novel myovirus E)
17	5	PT- LPs*	-
18	3	Siphovirus	53L (Defective myovirus B)
19	1	PT- LPs*	-
20	2	PT- LPs*	-
21	1	PT- LPs*	-
22	2	PT- LPs*	36L (No phage)
23	1	PT-LPs*	-

∗, Phage tail-like particles

Prophage carriage among the 91 *C. difficile* 027 induced using mitomycin C or norfloxacin was correlated to their multiple-locus variable number tandem repeat analysis (MLVA) types. MLVA 1-15 yielded defective myoviruses with three exceptions in MLVA 12 (66L yielding a siphovirus E), MLVA 13 (96L yielding a myovirus A) and MLVA 15 (91L yielding a myovirus A) in addition to the defective myoviruses. MLVA 16 and 17 and 19-23 all yielded phage tail-like particles (PT-LPs) except in MLVA 16 with one isolate yielding myovirus E and another (36L) in MLVA 22 which yielded no phage under the experimental conditions. Among the 3 isolates examined in MLVA 18, two yielded siphoviruses and one (53L) yielded defective myoviruse. Inductions were performed in duplicates on at least two separate occasions.

Table 3.2 Phage carriage in 91 *C. difficile* 027 isolates in relation to their different Pulsovar types.

Pulsovar type	Number of isolates	Morphology of phage induced	Exceptions
I	53	Defective myovirus	71L (PT-LPs) *
II	1	Defective myovirus	-
III	2	Siphovirus	-
IV	33	PT-LPs*	10 isolates (Myovirus A, E and defective myovirus B, C)
V	2	PT-LPs*	36L (No phage)

∗, Phage tail-like particles

Prophage carriage among the 91 *C. difficile* 027 induced using mitomycin C or norfloxacin was also correlated to their Pulsovar types. Pulsovar types I and II yielded defective myovirus with one exception in Pulsovar I (isolate 71L) which yielded only phage tail-like particles (PT-LPs). Pulsovar type III yielded siphovirus. Pulsovar types IV and V yielded phage tail-like particles with ten exceptions in Pulsovar type IV and one in Pulsovar type V. Inductions were performed in duplicates on at least two separate occasions

3.3.3   Norfloxacin and mitomycin C prophage induction

To optimise prophage induction, five concentrations (0.3, 1, 3, 6 and 9 µg/ml) of norfloxacin and mitomycin C were used to induce ten of the isolates. All of the induced cultures released prophages with 0.3, 1 and 3 µg/ml of antibiotic and there was an increase in phage particle density within this antibiotic concentration range. Although most cultures were poorly induced at 6 µg/ml concentration of either antibiotic, one sample (16L) produced the highest phage particle density at this concentration (Figure 3.5). All cultures were killed after 24 h of induction at 9 µg/ml and no phages were observed in these culture filtrates. It was also observed that morphologies of the induced phage particles did not vary with antibiotic concentration. It was therefore established that 3 µg/ml concentration of either antibiotic is optimal for *C. difficile* phage induction.
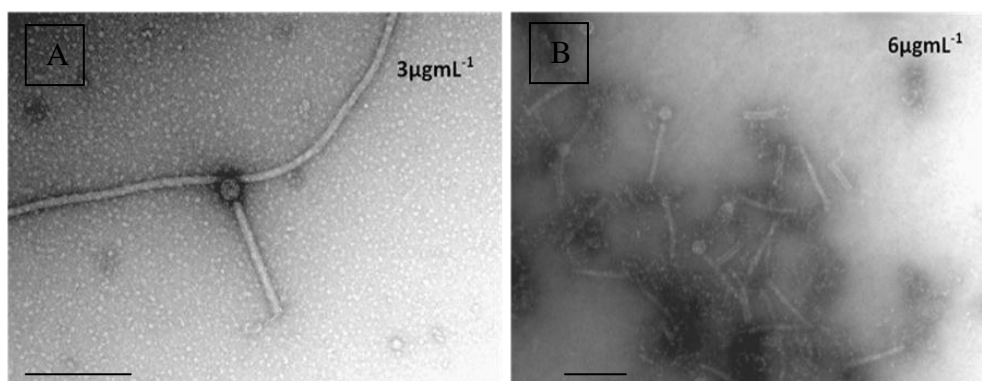
Figure 3.5 TEM images showing different phage particle density from induction of strain 16L.

A. Shows phage particle density with induction with 3 μg/ml and B. with 6 μg/ml concentration of mitomycin C. Scale bars represent 100 nm

The specificity of the two antibiotics to prophage induction was compared. It was observed that mitomycin C and norfloxacin were quite specific in their ability to induce prophages from their hosts. For example, sample 48L yielded two morphotypes of siphovirus with mitomycin C induction as discussed in section 3.3.1.2. However, with norfloxacin induction, isolate 48L yielded a single morphotype of siphovirus with tail length of ~320 nm (Appendix 2). In another instance one sample 66L, yielded two phage morphologies (siphovirus and defective myovirus) after induction with norfloxacin but only a defective myovirus with mitomycin C induction (Appendix 2). In addition, phages were only observed with mitomycin C induction of 16L while norfloxacin induction of this isolate yielded no intact phage except phage tail-like particles. Furthermore, isolate 96L yielded myoviruses and defective myoviruses when induced with norfloxacin and phage tail-like particles with mitomycin C. Finally, isolates 91L yielded myoviruses with mitomycin C but induction with norfloxacin yielded a myoviruses and defective myoviruses (Appendix 2).

The induction efficacy of the two antibiotics was also compared. This was done by counting the number of isolates that produced intact phage particles by each of the

antibiotic. It was observed that 55 (~60 %) of the strains could be induced by either norfloxacin or mitomycin C. However, only 5 strains (~6 %) could be induced by mitomycin C alone and 30 (33 %) by just norfloxacin. One strain (1 %) was not induced by either antibiotic (Figure 3.6).
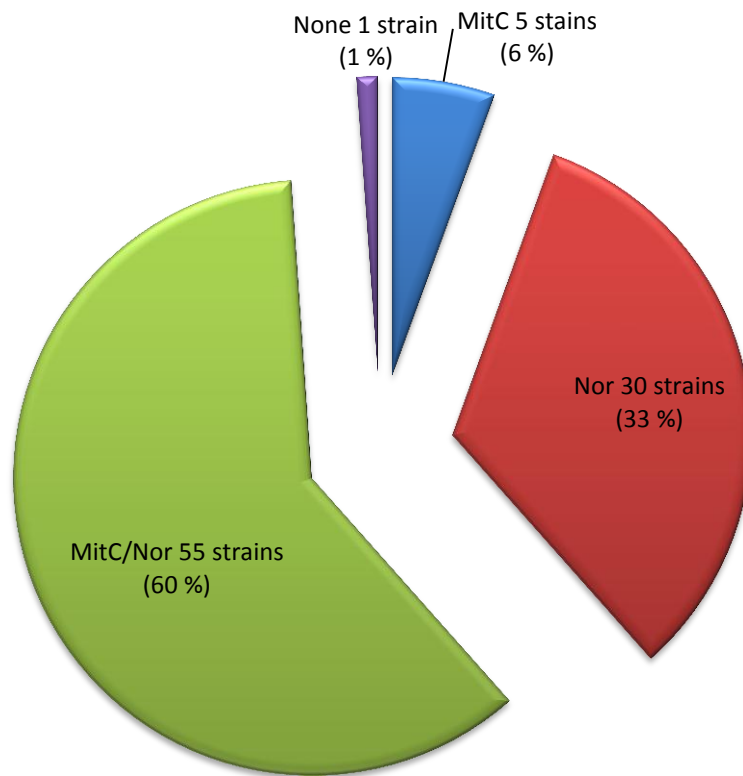

Figure 3.6 Pie chart showing proportion of *C. difficile* strains induced with different antibiotics.

The largest proportion of the isolates (60 %) was inducible by both antibiotics. Approximately 33 % and 6 % of the isolates were induced with norfloxacin and mitomycin C respectively. Only one isolate (1 %) was not induced by neither norfloxacin nor mitomycin C.

3.3.4 Growth patterns of induced cultures during 24 h induction

It was established from the previous experiments in section 3.3.3 that 3 μg/ml antibiotic concentration was optimal for prophage induction of the isolates used in this study. Therefore, all 91 cultures in BHI at OD_{550} ~1.2 were induced with norfloxacin or mitomycin C at this concentration for 24 h. The effect of antibiotics on the growth of bacterial cultures was determined by measuring the OD_{550} values before and after induction (Appendix 3). The growth responses of all the induced cultures all fitted into one of four patterns (A-D) and this was compared to phage release (Figure 3.7). Generally, it was observed that phage release did not correlate with the drop or rise in OD_{550} values. The four patterns of responses of the induced bacterial cultures are discussed below:

I.   For pattern A, there was a drop in OD_{550} with both mitomycin C and norfloxacin inductions. Although this pattern has previously been reported in *C. difficile* inductions, only 35 of the 91 isolates used in this study showed this profile. Furthermore, from the 35 isolates only 17 were found to harbour intact phages with the remaining 18 harbouring phage tail-like particles following induction by either antibiotic (Figure 3.7, Appendix 3)

II.   For pattern B, the OD_{550} dropped with mitomycin C induction but increased following norfloxacin induction. Twenty eight of the induced isolates showed this pattern. The TEM analysis showed that there was phage release from 16 and 11 of the isolates following mitomycin C and with norfloxacin induction respectively. One strain (36L) was found to contain no phages (Figure 3.7, Appendix 3).

III.   Pattern C was the opposite of pattern B with OD_{550} increasing following mitomycin C induction but dropping following norfloxacin induction. Only 5

isolates showed this pattern. There was phage release from 2 isolates with mitomycin C and 3 with norfloxacin induction as confirmed by TEM analysis (Figure 3.7, Appendix 3).

IV. For pattern D, the OD_{550} remained relatively constant following either norfloxacin or mitomycin C induction of the cultures. This pattern was observed in 23 samples. Only defective myoviruses and phage tail-like particles were observed with these strains (Figure 3.7, Appendix 3).
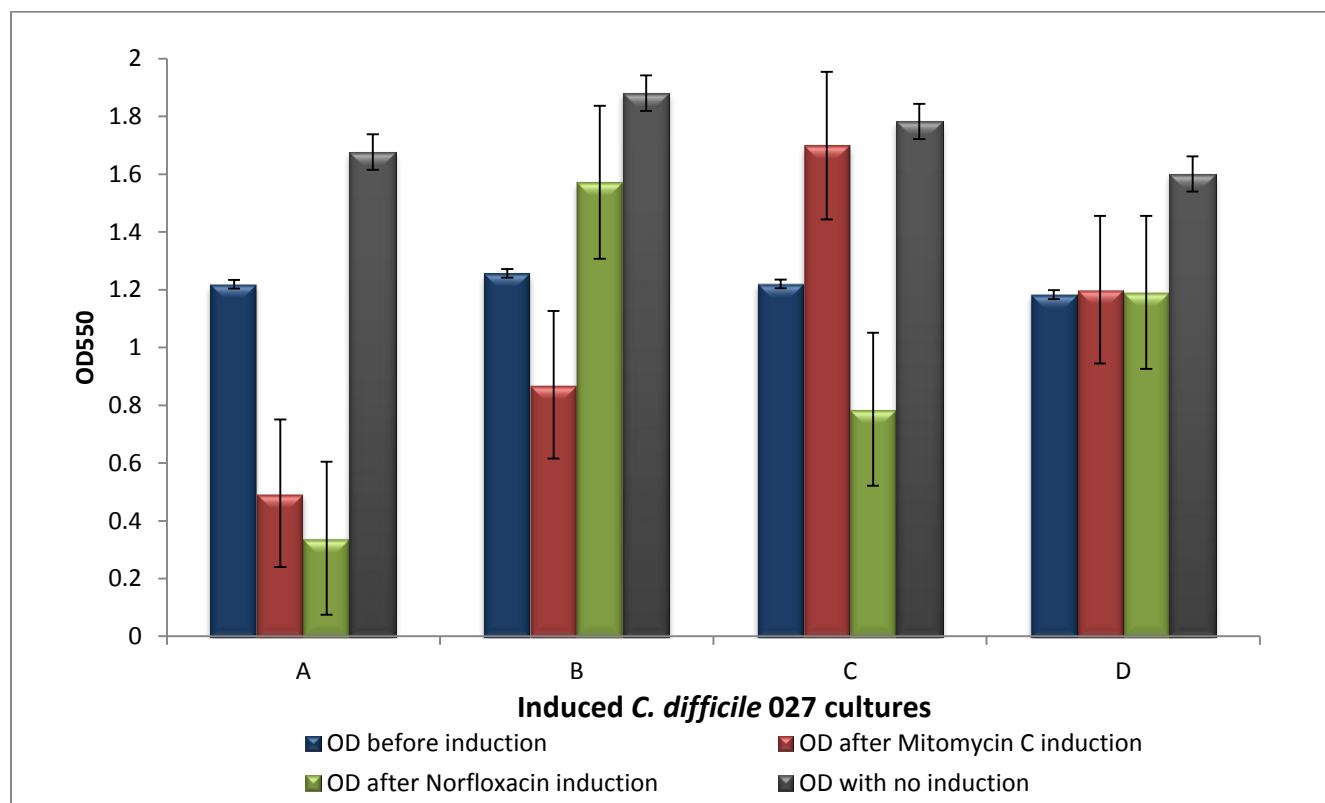
Figure 3.7 Graph showing different patterns of growth response of *C. difficile* cultures during prophage induction

Overnight cultures of *C. difficile* 027 isolates in BHI at OD_{550} ~1.2 were induced with norfloxacin or mitomycin C at a final concentration of 3 μg/ml for 24 h. Final OD_{550} were taken at the end of 24 h. The effect of antibiotics on the growth of bacterial cultures was determined by measuring the OD_{550} values before and after inductions. The growth responses all fitted into one of four patterns (A-D) and this was compared to phage release. Values represent means and standard errors. Experiments were carried out in duplicates and in at least two occasions. The effects of the inducing antibiotics on isolates representing the four patterns A –D cultures were extended for up to 120 h and are demonstrated in the figures 3.8-3.11.

3.3.5 The 120 h effects of norfloxacin and mitomycin C on induced cultures

In addition to the 24 h observations of the induced cultures, 3 strains each representating the four different patterns were selected and their induction time was extend to 120 h. The OD_{550} values were measured at additional time points of 48, 72, 96 and 120 h. The average of the OD_{550} values of the three strains at each time was computed and used as the value for that time point. In addition, 1 ml of the induced cultures were sampled at each time points and centrifuged and the phage particle density was analysed by TEM. It was observed that by the end of the experiment all the 12 strains showed a significant drop in OD_{550} values and a concomitant release of phages was observed as confirmed by the TEM analysis except in one strain 36L that showed no phage release from the initial 24 h induction (Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.11). Details of the 120 h effects of the antibiotics on the induced cultures belonging to the four different patterns are discussed below:

The 120 h effect of norfloxacin and mitomycin C on representative *C. difficile* cultures with pattern A is shown in Figure 3.8. A drop in OD_{550} with both mitomycin C and norfloxacin inductions was observed and this same effect was continued throughout the 120 h period. At the end of the experiment, the OD_{550} has dropped to 0.3 with norfloxacin and 0.1 with mitomycin C. There was increase in phage particle density as the time progressed as confirmed by the TEM analysis. However, one strain, 36L which fell under this growth pattern did not yield any phage during this time period. Bacterial cell death was observed after the 96 h as indicated by drop in the OD_{550} from ~1.5 to ~1.2 in the control culture.
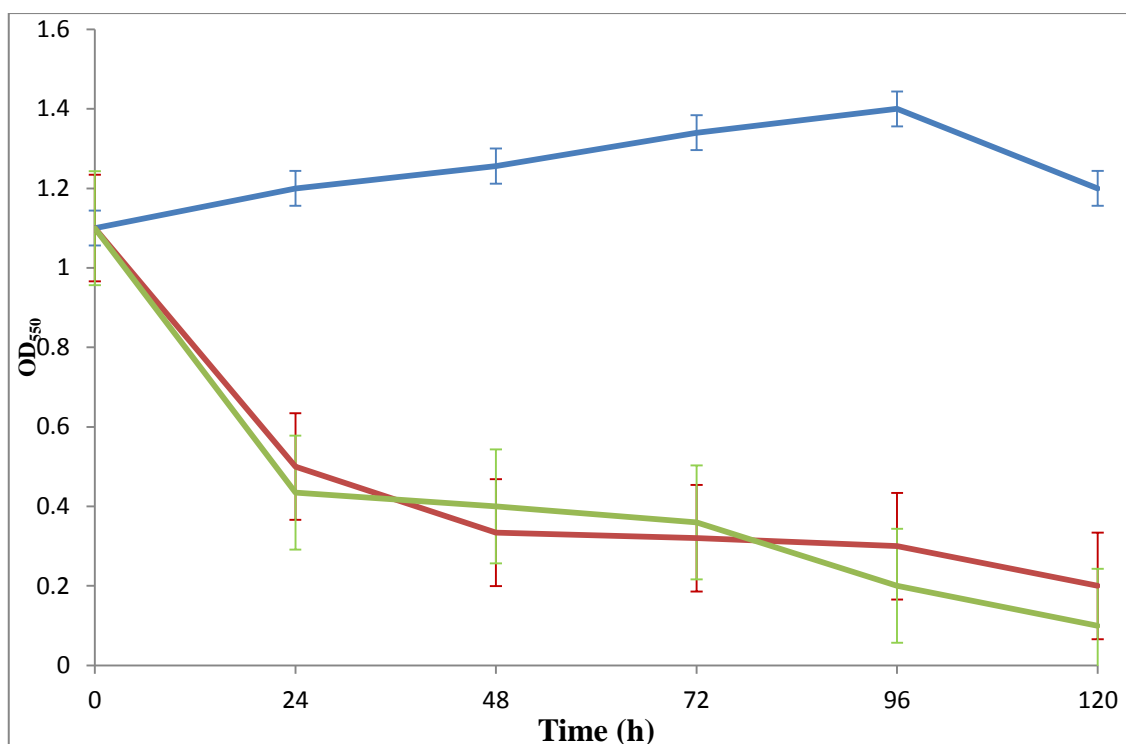
Figure 3.8 120 h effect of norfloxacin and mitomycin C treatments on growth of three induced _C. difficile_ cultures showing the pattern A

Above shows the effect of norfloxacin or mitomycin C on growth of three cultures 52L, 2L and 28L showing pattern A (Figure 3.7). Inductions were carried out for 120 h. OD_{550} was taken at 24 h interval following inductions and these were correlated to phage release. Values represent means OD_{550} values for the three induced cultures and standard errors. A drop in OD_{550} with both mitomycin C and norfloxacin inductions was observed from the 24 h and this same effect continued throughout the 120 h period. There was increase in phage density as the time progressed with both inductions for the three cultures. Experiments were carried out in duplicates and on two occasions. Control ━━━ Norfloxacin induction ━━━ Mitomycin C induction ━━━

In Figure 3.9**,** the 120 h effect of the norfloxacin and mitomycin C treatments for 120 h on representative strains with pattern B is shown. In this pattern there was an initial OD_{550} increased from ~1.18 to ~1.4 within the first 24 h of mitomycin C induction but decreased to 1.2 by the 48 h. After 48 h dramatic decrease in OD_{550} values to 0.6 and 0.4 at 96 and 120 h respectively was observed. Although phages were released irrespective of increase in OD_{550} in the 24 h time point, there was increase in phage particle density from the 48 h time point as confirmed by TEM which coincided with significant OD_{550} drop. However, with norfloxacin induction, a significant drop in

91

OD$_{550}$ from the first 24 h was observed. By 24 h of induction with norfloxacin, OD$_{550}$ dropped from of 1.18 to 0.6 and by the 72 h it dropped to 0.2. This value remained stable till the end of the 120 h (Figure 3.9). Although drop in OD$_{550}$ value was observed with norfloxacin induction from the first 24 h, there was no phage release from the induction as confirmed by the TEM analysis.
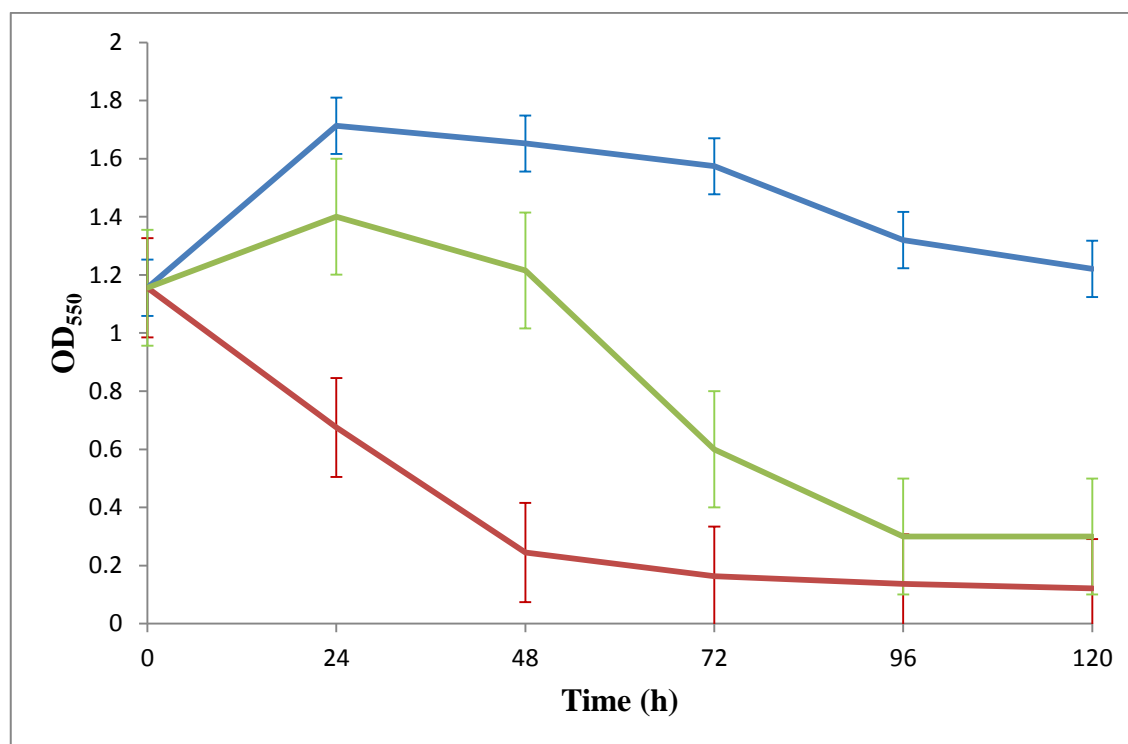

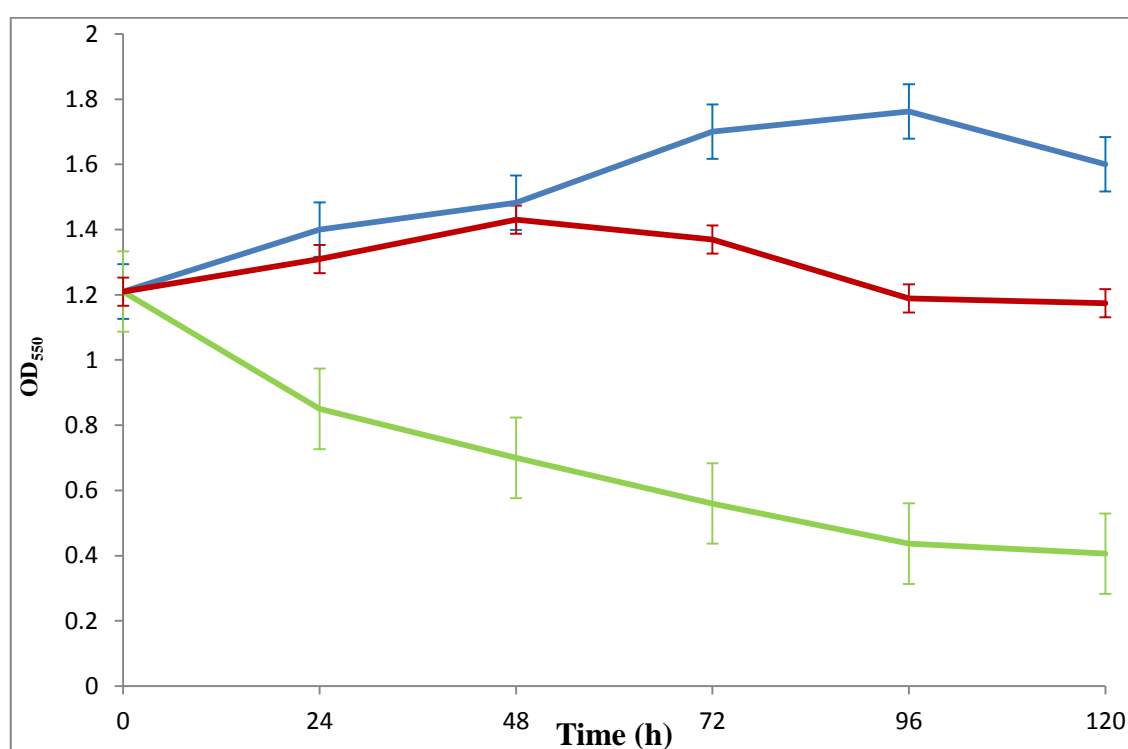**Figure 3.9 120 h effect of norfloxacin and mitomycin C treatments on growth of induced *C. difficile* cultures showing the pattern B**

The effects of norfloxacin or mitomycin C on growth cultures of 36L, 16L and 10L showing pattern B (Figure 3.7) are shown. Inductions were carried out for 120 h. OD$_{550}$ values were taken at 24 h interval following inductions and these were correlated to phage release. Values represent mean OD$_{550}$ values for the three induced cultures and standard errors. OD$_{550}$ value dropped from the first 24 h with mitomycin C. With norfloxacin, there was a rise in OD$_{550}$ value during the first 24 h but dropped afterwards. There was phage release with mitomycin C induction but not with norfloxacin with 16L and 10L but no phage release was observed with 36L with both inductions. There was also a significant rise in phage particle density with mitomycin C induction from the 48 h which correlated with the significant drop in the OD$_{550}$ value. Experiments were carried out in duplicates and on two occasions. Control ▬ Norfloxacin induction ▬ Mitomycin C induction ▬

Figure 3.10 shows the 120 h effect of the three strains which showed pattern C. It was observed that there was increased OD_{550} value from ~1.2 to ~1.4 by 48 h of induction with mitomycin C. The OD_{550} value dropped to ~1.2 by the 96th hour and remained at this value till the end of the experiment. Induction with norfloxacin showed a dramatic drop of OD_{550} to ~0.7 by 24 h of induction. By the end the 120 h experiment OD_{550} value had dropped to ~0.4. Although phages were released irrespective of the rise or drop in OD_{550} value within the first 24 h with both inductions, more phage particle density was observed after the 48 h as confirmed by the TEM analysis.

Figure 3.10 120 h effect of norfloxacin and mitomycin C treatments on growth of induced *C. difficile* cultures showing the pattern C

Above shows the effects of norfloxacin or mitomycin C on growth cultures of 38L, 12L and 78L showing pattern C (Figure 3.7) after induction for 120 h. OD_{550} values were taken at 24 h interval following inductions and these were correlated to phage release. Values represent means OD_{550} values for the three induced cultures and standard errors. There was an initial rise of OD_{550} with mitomycin C induction after the first 48 h but began to decrease from the 72 h. With the norfloxacin induction, there was OD_{550} decrease from the beginning to the end of the 120 h. Phages were released irrespective of the rise or drop or drop in OD_{550} values with both inductions in the three cultures. Experiments were carried out in duplicates and on two occasions. Control ——— Norfloxacin induction ——— Mitomycin C induction ———

In pattern D, OD_{550} values remained relatively constant up to the first 24 h with both antibiotics. After this period, the OD_{550} dropped to ~0.5 in both inductions (Figure 3.11). Phages were detected in all the samples during these time periods but there was increase in phage particle density from 72 h induction period.
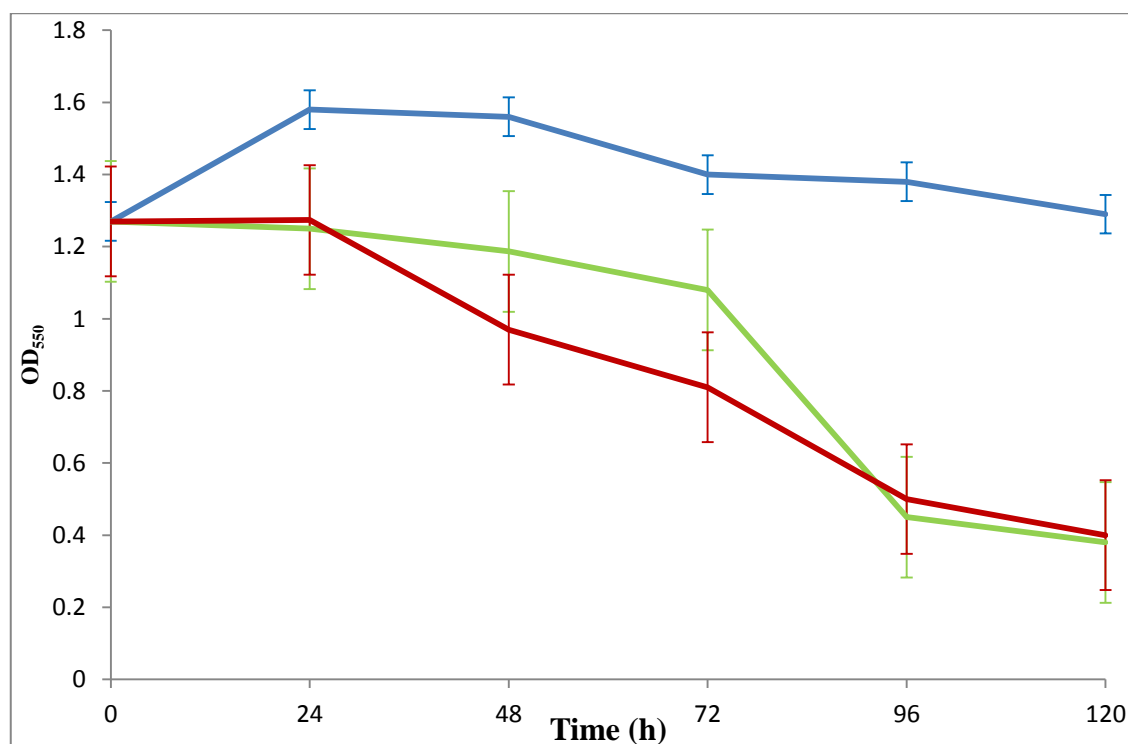

Figure 3.11 120 h effect of norfloxacin and mitomycin C inductions on growth of induced *C. difficile* cultures showing the pattern D

The effects of norfloxacin or mitomycin C on growth cultures of 32L, 77L and 26L showing pattern D (shown in Figure 3.7) after induction for 120 h are shown. OD_{550} was taken at 24 h interval following inductions and these were correlated to phage release. Values represent means OD_{550} values from the three induced cultures and standard errors. In this pattern of growth response, OD_{550} values remained relatively stable within the first 24 h with both norfloxacin and mitomycin C inductions but eventually began to drop from the 48 h till the end of the 120 h induction period. Phages were observed after 24 h treatment but increased phage density was observed after 48 h which also coincided with the observed significant drop in OD_{550} values in the isolates. Experiments were carried out in duplicates and on two occasions. Control ▬ Norfloxacin induction ▬ Mitomycin C induction ▬

3.3.6 Genetic diversity of *C. difficile* 027 temperate bacteriophages

The phage genome sizes were determined using PFGE analysis. Generally, PFGE has a practical limitation as it requires a large quantity of DNA (~200 ng) or phage particles of ~ 10^7-10^6 (Sambrook *et al.*, 1989, Larson *et al.*, 2000). Therefore, only ten samples showing high phage particle density and cut across the different phage morphologies as confirmed by the TEM analysis were selected for PFGE analysis. To obtain a high amount of DNA, a large scale induction of 1 l was carried out for each of the sample examined and the phage supernatants were purified using PEG. By this method, a DNA sample of ~120 ng/ml was obtained from each of the sample examined.

PFGE analysis revealed that phage genome size ranged from ~15 to 50 kb (Figure 3.12). The smallest genomes were observed in the defective myoviruses B and C from isolates 96LN2, 84LN, 91LN, and 66LM (lanes IV, V, VI and VII respectively) and the novel myovirus E (lane VIII). (Suffixes N and M symbolised isolates induced with norfloxacin and mitomycin C, respectively). The standard myoviruses A induced from isolates 96LN1 and 91LM (Lanes IV and IX respectively) had a genome size of ~30 kb as well as the siphoviruses induced from 37LM (lane II), 48LM2 (lane III) and 37LN (Lane X). Only the siphovirus induced from 48LM1 and the defective myovirus induced from 82LN had a large genome size of ~50 kb (lanes III and XI respectively). Two isolates (48LM and 96LN) previously shown to exhibit dual phage carriage exhibited genomes of two distinct sizes, 50 and 30 kb for 48LM1 and 48LM2 (lane III) and 30 and 15 kb for 96LN1 and 96LN2 (lane IV) respectively. This provides further evidence of their dual phage status. On the other hand, morphologically distinct phages that were induced from the same isolate but using different antibiotics showed different genome sizes (91LM with ~30 kb genome size and 91LN ~15 kb). Furthermore, in all cases examined, the siphoviruses which were morphologically identical under TEM

also showed identical genome sizes (for example 48LM2, 37LN and 37LM having genome size of ~30 kb).
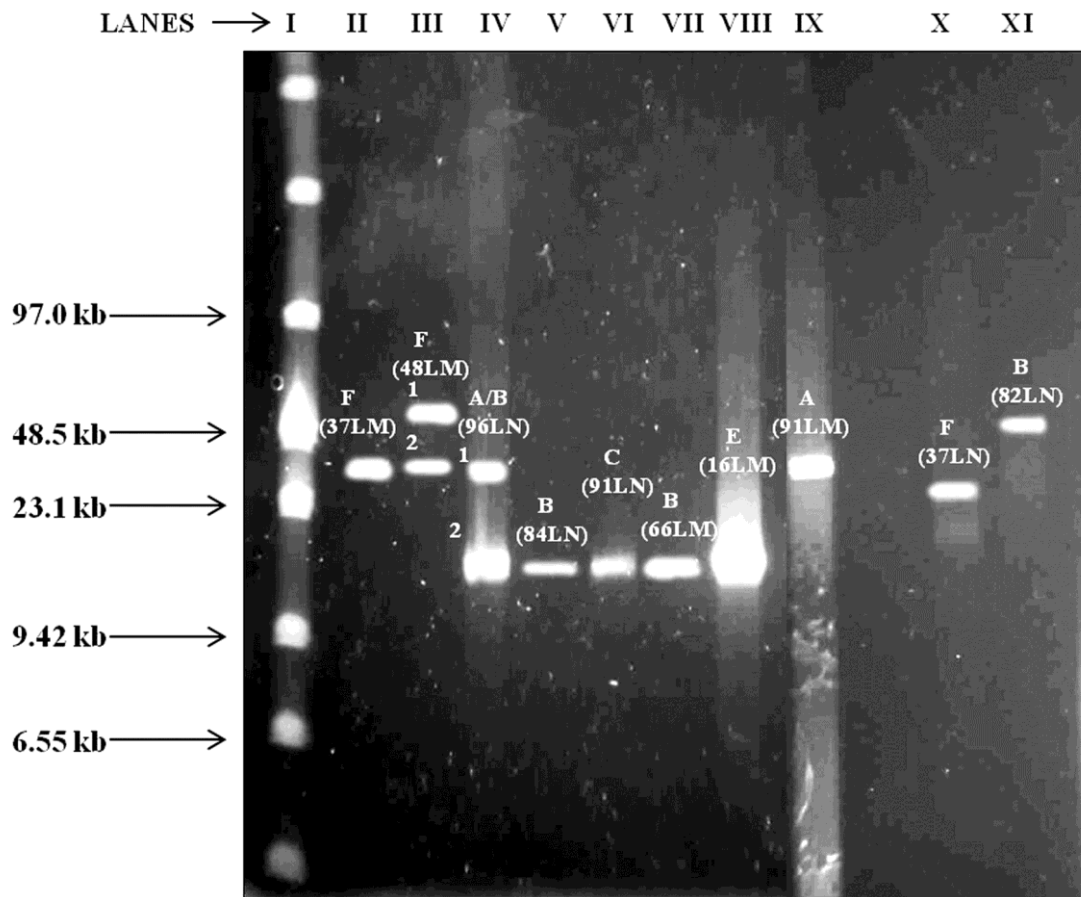

Figure 3.12 Pulsed-field gel electrophoresis analysis of whole temperate phage genomes showed diverse sizes.

Pulsed-field gel electrophoresis was used to analyse the PEG purified phage lysates. All the defective myoviruses with morphology B and C in samples 96LN2, 84LN, 91LN, 66LM and novel myovirus E 16LM had genome size of ~15 kb. The siphovirus F in 48LM2 and 37LM and typical myovirus A in samples 96LN1 and 91LM had genome size of ~30 kb. Only the siphovirus in 48LM1 and defective myovirus in 82LN had genome size of ~50 kb. Samples with suffix N, M indicates isolates induced with norfloxacin or mitomycin C respectively.

3.3.7 Host range of temperate bacteriophages of *C. difficile*

Several attempts were made to propagate the phages induced from isolates induced in this study using plaque assays and spot tests. The induced phages were tested on 63 *C. difficile* hosts which consisted of 18 environmental and 45 clinical isolates. Unfortunately, none of the phages could infect these hosts in a lytic way. However, there were some phages capable of partial lysis. For example one phage induced from isolate 16L could lyse CD630 (Figure 3.13). This phage was a myovirus (myovirus E) with a small capsid (Figure 3.1). The plaques produced were very small of about 1 mm in size (Figure 3.13). Interestingly, the plaques formed from the infection of this phage on CD630 could not propagate further and TEM analysis could not identify whole phage particles from these lysates.
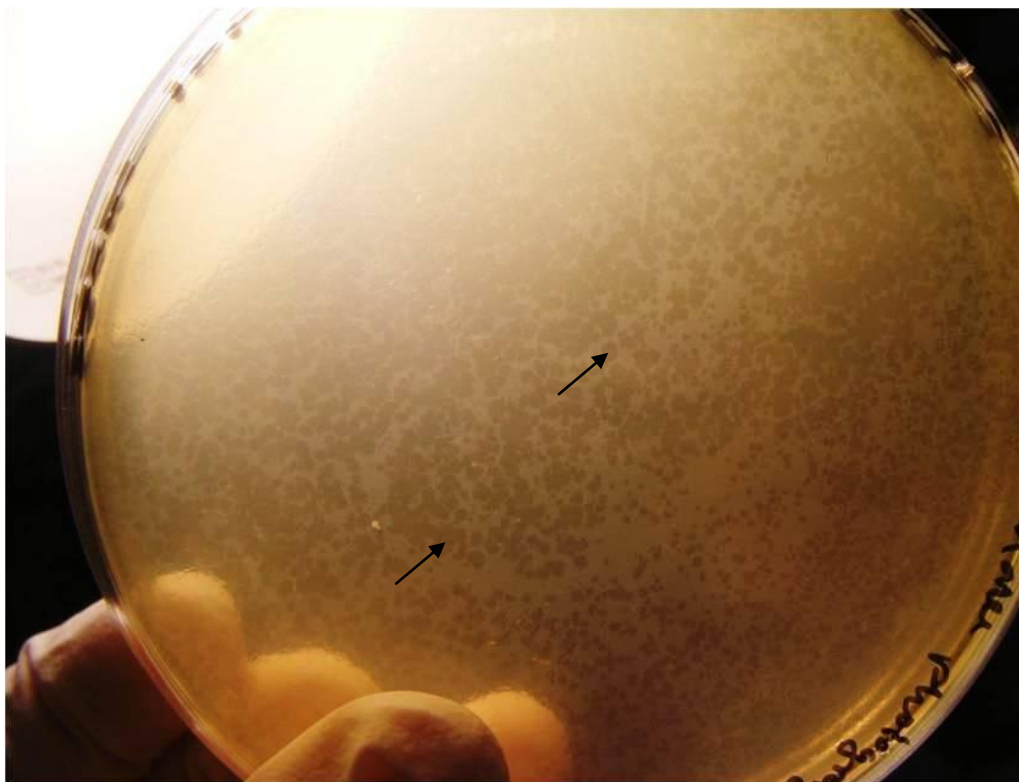
Figure 3.13 Picture showing plaques of myovirus E on lawn of CD630

Above is a picture of a BHI plate showing plaques (indicated by black arrows) that resulted following induction from 16L and plaque assay on a lawn of CD630. The plaques obtained could not propagate further in a lytic way and no phages were identified from the scrapped plaques in SM buffer.

The protocol for plaque assays and spot tests were optimised on a phage which did have a host to try and get the phages to propagate. One of the factors modified during the plaque assay optimisation included different concentrations of agar in the BHI top layer. However, it was observed that whether 0.4, 0.6 or 0.7 % agar w/v agar used in this layer did not alter plaque formation. Furthernore, agar top layer concentration did not alter the size of the plaques of 16L on CD630.

In addition to the top agar concentration, salt supplements were added to the BHI semi solid agar as previously reported (Mahony *et al.*, 1985). Again this did not extend the host range of the phages. It was thought that the induced phage lysates may be too diluted to form plaques or that there may not be phages present. Therefore, plaque assays were also carried out with PEG purified phage suspensions. TEM examination following PEG purification showed that the phage particle density had increased but still none of these phages could infect these hosts. It is known that PEG treatment could significantly reduced the infectivity of phages (Bermudez and Hubbell, 2005). However, this may not apply to the phages induced in this study as phage 16L could still produced plaques on CD630 lawn after PEG purification.

It was suspected that the infectivity of the phages could be affected by the prophage induction method. The phages identified in this study were induced from *C. difficile* cultured in plain BHI broth using norfloxacin or mitomycin C. Therefore, an alternative procedure of prophage induction with mitomycin C as described by Goh *et al*. (2007) was adopted. In this method *C. difficile* isolates were cultured in BHI broth supplemented with 5 % horse blood, 50 mg/ml of erythromycin and 10 mg/ml of tetracycline before induction with with mitomycin C at 3 mg/ml (Goh *et al*., 2007). Therefore, eight selected *C. difficile* isolates (91L, 16L, 96L, 66L, 48L, 82L, 84L and 52L) were induced as described Goh *et al*. (2007). The putative phage stock recovered

after centrifugation was used in plaque assays and spot tests as descibed previously. In this instance, clearance following spot tests was produced by the eight phages on lawns of five different environmental hosts. The clearance produced was observed to diminish with phage dilution. Although phages were observed in the scraped zones of the clearance, the suspension could not clear fresh culture of hosts. Furthermore, none of these phages could produce descrete plaques on the lawn of their hosts except 16L which cleared lawn of CD630.

Since the plaque assays and spot tests were carried out in a semi-solid agar which may hinder phage migration, an alternative method in broth culture was attempted. Approximately 5 ml of bacterial cultures at OD_{550} were infected with 500 μl-1 ml of the phage supernatants. Unfortunately, the bacterial cultures did not show decrease in growth to indicate phage lysis and no phage could be detected in the infected cultures using TEM examination. Phage enrichment in broth culture was also attempted. In this procedure, the phages were inoculated in a bacterial host culture for 24-48 h and the resultant filtered supernatants were used in plaque assays and spot test as described in section 2.6.2. In addition to the phage enrichment, two or more phage supernatants were co-cultured with a host before carrying out plaque assays. Neither the phage enriched supernantants nor the co-culturing methods could yield further plaques with plaque assays and spot tests.

It is clear that these phages have a very narrow host range and therefore identifying host for them is extremely difficult.

3.3.8 Enzyme restriction profiles of *C. difficile* phages

Several attempts were made to characterise the induced phages according to their enzyme restriction profiles. Ten different restriction enzymes were used to digest the phage DNA samples according to the manufacturers' instructions. The enzymes

used included seven 6-base pair enzymes (BamH1, Nde1, BstB1, HindIII, EcoR1, Sty1 and Sma1) and three 4-base pair enzymes (Sau3A1, Mbo1 and Dpn1). The enzymes Sau3A1, Mbo1, Dpn1 and BstB1 are known to cut methylated DNA. Unfortunately, none of these restriction enzymes used could digest the DNA from the induced phages, or if they digest the DNA, it was only partial so they did not result in suitable banding patterns to allow genomic comparisons.

3.4 Discussion

There is an ongoing debate on the nature and existence of the hypervirulence of *C. difficile* 027 strains. Recent publications suggest that 027 strains are associated with varying disease severities (Morgan *et al.*, 2008, Goldenberg and French, 2011, Sirard *et al.*, 2011). These studies also showed that the commonly used markers of the 027 strain such as 18 bp and 1 bp deletions in the *tcd*C and the carriage of the binary toxin genes do not always correlate with disease severity or outcome of *C. difficile* infections. Thus, identifying other factors associated with specific 027 subtypes and linking this to disease severity is of clear interest to resolve this issue. The factors identified could also potentially explain why some isolates produce more disease cases than others and the apparent inconsistencies in epidemiological studies.

In this study, I investigated the possibility that prophage carriage may be a factor that may contributes to the diversity and virulence of *C. difficile* 027 strains. Bacteriophage carriage within a single *C. difficile* ribotype has not been extensively examined in any ribotype. The only study on phage carriage at the time of this study examined six isolates from different *C. difficile* ribotypes and found that each ribotype harboured a morphologically and genetically different prophage and suggested a correlation between the type of prophage and ribotype (Fortier and Moineau, 2007). However, my previous finding shows that diverse phage morphologies are associated with the ribotype 027 (Nale, 2009). Furthermore, the examination of 91 ribotype 027 isolates in this study show that prophage carriage within *C. difficile* 027 is morphologically and genetically variable and therefore contributes to diversity (Nale *et al.*, 2012).

The morphology of the putative myovirus A (Figure 3.1) identified in this study is similar to the temperate phage induced from clinical and environmental isolates of *C.*

difficile 027 and to temperate phages found in unknown ribotypes (Nagy and Foldes, 1991, Goh *et al.*, 2005a, Goh *et al.*, 2005b). Prior to this study, this is the only temperate bacteriophage morphology previously reported to be associated with 027 strain (Fortier and Moineau, 2007). However, despite the capsid and tail diameters being similar to the previously described phage, the viruses described here all have longer tail lengths suggesting that they may be of a different origin (Mahony *et al.*, 1985, Goh *et al.*, 2005b, Fortier and Moineau, 2007). The defective myoviruses B, C and D (Figure 3.1) observed from the majority of the isolates have also previously been shown to be associated with mitomycin C induced lysates which also contain typical myoviruses (Mahony *et al.*, 1985, Goh *et al.*, 2005b, Fortier and Moineau, 2007). However the majority of lysates examined here only had these 'defective' particles. Although it is assumed that phage tail sheaths contract when infecting a host and ejecting DNA, TEM analysis carried out in this study did not confirm this. This data therefore, further support previous observations that *C. difficile* can carry two morphologies of myoviruses (Goh *et al.*, 2007).

The novel *C. difficile* defective myovirus E identified from one isolate had an unusual small capsid compared to other normal *C. difficile* phages (Figure 3.1). This phage is similar to 'killer particles' of *Bacillus* species, *Acetobacter* species and *Clostridium botulinum* (Bradley, 1965, Inoue and Iida, 1968, Sunagawa and Inoue, 1991). These kind of phage particles are thought to package host DNA instead of bacteriophage DNA and are able to kill sensitive hosts but not replicate within them (Bradley, 1965, Okamoto *et al.*, 1968). Attempts to propagate this phage on lawns of many *C. difficile* isolates in this study showed that it could clear lawns of only one host (CD630) but the scraped zones of killing (plaques) could not further propagate and only broken tails instead of intact phages could be identified in the scraped lysate. This is

particularly interesting as the CD630 has been reported to contain two prophages in its genome (Goh *et al.*, 2007) and this should confer immunity to it against phage super-infection (Scott, 1975). Further TEM investigation revealed that intact phages could not be identified in the lysates of the plaques formed from this phage. Therefore, it can be suggested that these phages have not actually replicated within the CD630 cells as suggested by Bradly (1965) and Okamoto (1968) and the plaques obtained could have occurred as a result of lysis from without as previously reported (Koch, 1964). Lysis from without is a phenomenon that describes a type of baterial lysis that occurs due to adsorption and penetration of a number of phages from outside the host cell. The destruction of the host cell wall by the penetrating phages and not a cycle of viral infection could have led to the lysis of CD630 (Ralston and McIvor, 1964). The ability of myovirus E to lyse a clinical *C. difficile* strain can potentially be exploited in the area of phage therapy for *C. difficile* infection.

Other temperate bacteriophage morphology identified here are the siphoviruses typified by F1 and F2 (Figure 3.2). Although, siphoviruses with dimensions similar to those induced here have previously been reported to be present within the genome of other ribotypes of *C. difficile*, this report is the first to show the association of this phage morphology to ribotype 027 (Mahony *et al.*, 1985, Nagy and Foldes, 1991, Fortier and Moineau, 2007, Sekulovic *et al.*, 2011). Interestingly, two of the three siphoviruses observed were induced from isolates within MLVA 18 which was associated with very high severity of CDI (death within the first thirty days of infection) (Fawley *et al.*, 2008). This suggests their possible role in the pathogenicity of 027 strains. Furthermore, a previous report has shown that infection of *C. difficile* ribotype 027 by phage CD38-2 (a temperate *C. difficile* siphovirus) resulted in the increase expression of toxin A and B (Sekulovic *et al.*, 2011). Although a bioinformatic analysis

of phage CD38-2 failed to identify toxins or virulence factors in the viral genome, the phage has been shown to interact with the *C. difficile* pathogenicity locus (PaLoc) thus having the potential to influence toxin production (Sekulovic *et al.*, 2011). A similar observation was made when temperate bacteriophages were induced and characterised from *C. difficile* isolates obtained from University Hospitals of Leicester (Tromans *et al.*, 2010). When the result of the phage carriage was correlated with the patients records, it was observed that there was an association between the presence of temperate siphoviurses of specific capsid size (45-55 nm) and an increased relative risk of 30-day all cause mortality (Tromans *et al.*, 2010). Therefore, phage detection may represent a useful marker to identify strains that are associated with increased disease severity and this early detection can direct treatment and patient management.

Dual phage carriage has previously been reported in *C. difficile* strains CD630, CD8 and CD38 in which two morphologically and genetically distinct phages were induced from a single *C. difficile* strain (Fortier and Moineau, 2007, Goh *et al.*, 2007). This study however, is the first to show two morphologically distinct phages with different genome sizes induced from a single ribotype (ribotype 027) strains. The correlation of phage carriage with MLVA types of ribotype 027 suggests that phage carriage is stable within this ribotype and does contribute to its diversity (Henn *et al.*, 2010). The acquisition of mobile genetic elements many of which encode genes for toxin and antibiotic resistance, suggests the potential role of temperate bacteriophages in the severity of *C. difficile* infections (Sebaihia *et al.*, 2006).

The presence of phage tail-like particles, such as those with morphology G in all the induced lysates, has previously been reported to be found in several induced lysates of *C. difficile* (Figure 3.3) (Nagy and Foldes, 1991, Fortier and Moineau, 2007). However, the lengths of the observed phage tail-like particles in this study were longer

than those in previous reports (an average size of 160 nm as opposed to 130 nm). These phage tail-like particles morphology are similar to phage tail-like bacteriocin of *Budvicia aquatica* and *Pragia fontium* and phage tail-like particles of *Vibrio spp* (Smarda and Benada, 2005, Gnezda-Meijer *et al.*, 2006). Although the particles have been induced from *C. difficile* isolates, their biological functions have not yet been determined. However, they were found to be present in all the induced isolates (in addition to other phage morphology), the only phage particles present in MLVA 16 isolates and the majority of induced isolates in Pulsovar IV. Epidemiological work indicated that isolates in MLVA 16 and Pulsovar IV were associated with early deaths (Fawley *et al.*, 2008). These particles could potentially be contributing to the virulence of their hosts, or providing some competitive advantage for their survival, as previously suggested (Casjens, 2008). The other phage-like particles with morphology H found in two of the mitomycin C induced samples, have a morphology that has not previously been observed to be associated with *C. difficile*. However, the phage particles H do closely resemble a bacteriocin induced from *Pseudomonas aeruginosa* p28 (Takeya *et al.*, 1969, Onishi *et al.*, 1971, Lee *et al.*, 1999, Smarda and Benada, 2005). Some of the bacteriocins have been shown to have bactericidal activities and provide competitive advantage to their hosts (Uratani and Hoshino, 1984, Casjens, 2008).

Norfloxacin and mitomycin C cause prophage induction in bacteria through DNA damage. Norfloxacin (a fluoroquinolone) achieves this by inactivating the action of DNA gyrase and topoimerase IV and disrupts DNA supercoiling (Kimmitt *et al.*, 1999, Matsushiro *et al.*, 1999, Kimmitt *et al.*, 2000). In contrast, mitomycin C (an azaridine) acts as a DNA alkylating agent and leads to mispairing of bases, DNA strand breakage, and cross-linking of complementary strands (preventing DNA synthesis) in bacteria (Paolo, 1982, Kimmitt *et al.*, 2000). In *E. coli*, DNA damage induces an SOS response,

a process of DNA repair. During the SOS response, RecA assumes a co-protease activity in the autocatalytic cleavage of the phage repressor protein resulting in the release of phage DNA (Kimmitt *et al.*, 2000, Janion, 2008). Although mitomycin C is the only antibiotic previously used for *C. difficile* prophage induction, norfloxacin has been successfully used in *E. coli* prophage induction (Mahony *et al.*, 1985, Nagy and Foldes, 1991, Goh *et al.*, 2005b, Fortier and Moineau, 2007). The specificity of the two agents in inducing prophages from their hosts as observed in this study demonstrates that their mode of action has a huge impact on the type of prophage induced. Consequently, exclusive use of mitomycin C can severely underestimate the number of inducible bacteriophages associated with *C. difficile*. It was observed that 90 of 91 isolates used in this study were found to harbour prophages or phage tail like particles. Approximately 33 % of these isolates would not have been induced if mitomycin C only had been used. This data are consistent with genome sequencing based analysis that showed that the majority of *C. difficile* isolates contain prophages or prophage-like elements in their genomes (Henn *et al.*, 2010).

To release their prophages, *C. difficile* cultures were induced using antibiotics at their mid-log phase. The mid-log phase was determined from my previous MSc work and ranged between 0.7-1.300 (Nale, 2009). Although other researchers have induced prophages from *C. difficile* at the stationary growth phase (Fortier and Moineau, 2007), others have performed this using just a 24 h culture (Sell *et al.*, 1983, Goh *et al.*, 2005b). In this study, the prophage inductions were carried out during the mid-log phase because the cells are at their greatest activity then, constantly in replication and, consequently, more susceptible to induction.

The four different growth patterns of bacterial cultures in response to the two antibiotic inductions show that different *C. difficile* strains respond differently to equal

stress exerted by these antibiotics. The increase in OD_{550} value after induction as observed in this study was also reported by (Hendry *et al.*, 1976). Although a fall in OD_{550} is often attributed to the lysis of bacterial cells due to prophage release (Fortier and Moineau, 2007), no correlation between this and phage release was observed as revealed by the electron microscopy. Phages were released whether there was a decrease or increase of OD_{550} value after induction but a dramatic release of phages coincided with significant decrease in OD_{550} values as confirmed by the TEM. It could be that the time taken for a *C. difficile* cell to undertake the SOS response leading to excision and release of phage may vary from one strain to the other. This variation may have resulted to the observed OD_{550} drop at various times during induction. In addition, just as lysogeny allows the phage to select its host and to provide an optimum environment for replication, so phages could escape from a host whose survival is being threatened (Dimmock *et al.*, 2007) (Dimmock *et al.* 2007). As the death of the host is eminent, the phages finally lyse their hosts leading to dramatic drop in OD_{550} values and massive release of phages from the 48 h hour following induction. Another reason for the delay in lysis may be due to lysis inhibition as observed in T4 bacteriophages (Paddison *et al.*, 1998). This is a situation in which T4 phage and its T-even family are able to control the timing of lysis in response to the relative availability of bacterial host in their environment. However, this has not been established in *C. difficile* phages.

It is evident from the results of this study that the temperate phages induced also have diverse genome sizes. The DNA size of ~15 kb (96LN2, 84LN, 91LN and 66LM) observed here are amongst the smaller DNA sizes for phages isolated from *Clostridium* species (Jones 2005). The genome sizes of temperate bacteriophages from isolates 91LM, 96LN1 (myoviruses) and 82LN (defective myovirus) are in agreement with the those of temperate bacteriophages of *C. difficile* belonging to *Myoviridae*, having

genomes varying in size from 29-160 kb (Jones, 2005, Govind *et al.*, 2006, Goh *et al.*, 2007). The genome sizes of the siphoviruses induced from 37L and 48L are also in agreement with previous report (Horgan *et al.*, 2010, Sekulovic *et al.*, 2011).

Despite several attempts to amplify the phages induced in this study through plaque assays and spot tests, none of the host strains were susceptible to infection. In addition, none of the phages could be digested with any of the restriction enzymes used. These findings further indicate the increasing problems associated with characterising *C. difficile* phages as previously suggested (Mahony *et al.*, 1985, Govind *et al.*, 2006, Goh *et al.*, 2007). The inability of the phages to infect the hosts or to be digested by the restriction enzymes used may be attributed to DNA methylases which are found in the genomes of *C. difficile* strains and which provide immunity to the restriction enzymes (Govind *et al.*, 2006, Sebaihia *et al.*, 2006). The modification of phage DNA by methylation has been reported in bacteria and could provide opportunity for adaptation for a phage population so that it could no longer be constrained by the action of hosts restriction endonucleases (Perret *et al.*, 2004).

3.5   Conclusion

Clearly, *C. difficile* 027 isolates contain morphologically and genetically diverse sets of bacteriophages that can be induced with specific antibiotics. The carriage of different bacteriophages by *C. difficile* 027 isolates shows that there is significant variation within the mobile genetic elements associated with this ribotype. The correlation of phage carriage with MLVA and Pulsovar types within *C. difficile* 027 also suggests that these bacteriophages contribute to its diversity.

3.6   Future work

Future work will aim to determine how bacteriophages contribute to the spectrum of disease severity observed with different *C. difficile* ribotypes. In addition, genome sequencing and host range determination are required to explore the possible role of these bacteriophages in the pathogenesis of CDI. More investigations are also needed to further characterise novel myovirus E and its potentials for phage therapeutic purpose.

4 Development of Molecular Markers for the Identification of *Clostridium difficile* phages

4.1 Introduction

Whole genome sequencing has offered insights into the evolution of *C. difficile* and showed that the organism is genetically diverse with a highly plastic genome that encodes several mobile genetic elements including transponsons and prophages (Sebaihia *et al.*, 2006, Stabler *et al.*, 2006, Goh *et al.*, 2007, Stabler *et al.*, 2009, Henn *et al.*, 2010). Temperate bacteriophages play important roles in the evolution and diversity of bacterial genomes by acting as agents of horizontal gene transfer (James *et al.*, 2001, Canchaya *et al.*, 2003, Hazen *et al.*, 2010, Hall *et al.*, 2011). The *C. difficile* temperate bacteriophages have been reported to affect the physiology of their hosts by supplementing and modifying toxin production (Goh *et al.*, 2005a, Govind *et al.*, 2009, Sekulovic *et al.*, 2011). Recently, temperate bacteriophages have been shown to contribute to the diversity of strains within *C. difficile* ribotypes (Nale *et al.*, 2012, Shan *et al.*, 2012).

Previous reports have characterised *C. difficile* temperate bacteriophages according to their morphology (using transmission electron microscopy), genome size (by pulsed field gel electrophoresis), enzyme restriction profile, DNA southern hybridization, protein analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and dot blots (Mahony *et al.*, 1985, Nagy and Foldes, 1991, Goh *et al.*, 2005b, Nale *et al.*, 2012, Shan *et al.*, 2012). Although these methods have revealed useful information, they are however labour intensive, cannot give genetic information and may require specific conditions. One of the factors limiting phage study is the choice of inducing agents (Nale *et al.*, 2012). Also, PFGE has been

shown to be non-reproducible and requires high phage titre which is not always obtainable with induced samples (Heng *et al.*, 2009). Finally, many *C. difficile* phages have very narrow host range which makes them extremely difficult to be propagated by plaque assays (Mayer *et al.*, 2008, Abhilash *et al.*, 2009). The inability of the phages to propagate through plaque assay leads to low phage particle density and consequently low DNA concentration. Characterisation of phages through restriction enzymes digestion is cumbersome in part due to DNA methylases identified in many genomes of *C. difficile* which helps protect the phages from their host enzymes (Govind *et al.*, 2006, Sebaihia *et al.*, 2006, Dong *et al.*, 2010, Enikeeva *et al.*, 2010). These problems can be circumvented by the use of a molecular marker.

At least 17 ribotypes of *C. difficile* contain prophages which can be induced using different inducing agents (Fortier and Moineau, 2007, Goh *et al.*, 2007, Shan *et al.*, 2012). However, only five temperate *C. difficile* bacteriophages have fully been characterised (Govind *et al.*, 2006, Goh *et al.*, 2007, Mayer *et al.*, 2008, Horgan *et al.*, 2010, Sekulovic *et al.*, 2011). The reasons for this very small number are due to the inherent difficulties in studying *C. difficile* phages. Therefore, the few phages characterised in *C. difficile* may represent a very small fraction of the so many *C. difficile* prophages that exists in their genomes (Henn *et al.*, 2010, Nale *et al.*, 2012, Shan *et al.*, 2012). Thus, there is a desperate need to develop alternative approaches to identify and study the diverse majority of *C. difficile* phages that are not amenable to existing study methods. Clearly, the most flexible and useful method to screen and study phage diversity is the design of primers to target specific phage conserved genes. Two conserved genes commonly used in the study phage diversity are the *gp20* and *gp23*, which encode for the capsid and portal genes respectively (Fuller *et al.*, 1998, Short and Suttle, 2005). The primers targeting these genes can be used as molecular

markers for the phage identification (Chang *et al.*, 2010). Unfortunately, there are no described molecular markers for the identification of *C. difficile* phages.

When the genomes of two sequenced *C. difficile* myoviruses phiC2 and CD119 and the prohages of CD630 and QCD-32g58 were analysed, their major capsid, portal and holin genes appeared to be conserved in part and variable in part to allow primer placement possible (Figure 4.1) (Goh *et al.*, 2007).
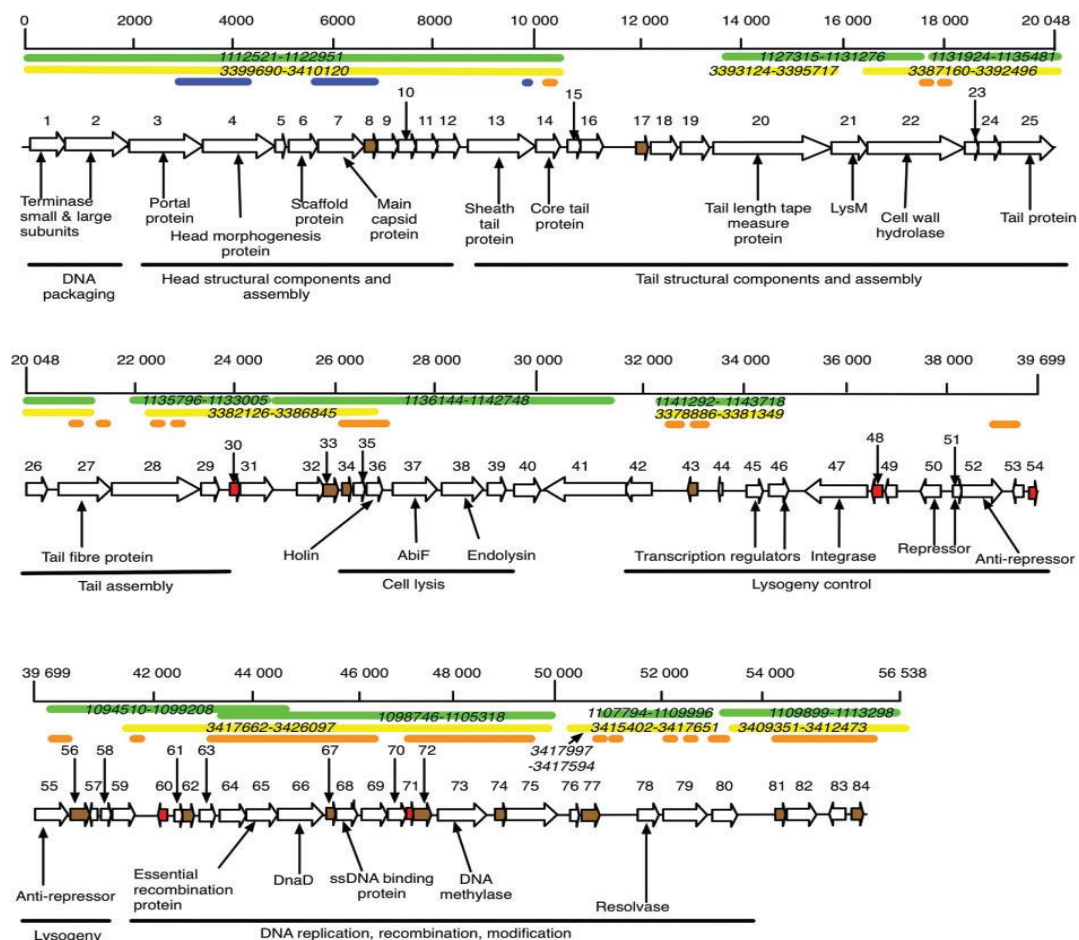
Figure 4.1 Genome sequence of phiC2 in comparison to prophages 1 and 2 of CD630, phiCD119 and QCD-32g58 (Goh *et al.*, 2007).

The direction of transcription and predicted ORFs of phiC2 are represented by black arrows. Their putative functions are indicated accordingly. The organisation of the genome is broadly categorised into six genetic modules namely: DNA packaging, head structure component and assembly, tail structure component and assembly, cell lysis, lysogeny control and DNA replication, recombination and modification. Nucleotide homology of prophage 1 and 2 of CD630 are shown in green and yellow bars respectively. Orange and blue bars represent regions of nucleotide similarity to CD119 and QCD-32g58, respectively. ORFs unique to phiC2 are in red, ORFs common to phiC2, phiCD119 and prophages in CD630 are in brown.

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Other genes that also appeared to have a useful amount of conservation include the core tail protein and the genes responsible for DNA replication, recombination and modification (Figure 4.1). Although the DNA polymerase gene has previously been used to study phage diversity within the podoviruses and myoviruses, this gene has not been detected in sequenced *C. difficile* phages, and hence, cannot be used for this purpose. (Chen and Suttle, 1996, Chen *et al.*, 1996, Fuller *et al.*, 1998, Goh *et al.*, 2007). Therefore, three conserved regions within the *C. difficile* phage genomes including the capsid, holin and portal protein genes were selected for the design of molecular markers to study phage diversity within *C. difficile.* Degerate PCR primers targeting the *C. difficile* phages were desigened based on the 5' and 3' ends of the aligned genes. PCR products from the amplification of these genes using these primers were sequenced and analysed using different algorithms to reveal diversity within the phages.

4.2 Aims of study

The aims of this chapter are to:

1. design molecular markers for the identification of *C. difficile* myoviruses

2. determine the phylogenetic relationships within *C. difficile* phages

4.3 Results

4.3.1 PCR ribotyping

As earlier stated in section 2.2.1, all the 91 *C. difficile* 027 strains used in this studies were previously characterised using PCR ribotyping. However, six randomly selected strains were re-ribotyped using the same primers used for the previous PCR (Stubbs *et al.*, 1999, Fawley *et al.*, 2008). This is to ensure that the isolates were not contaminated or compromised in any way during the isolation procedures from the paper discs. The resultant ribotype patterns were visualised on agar gels and all the six randomly selected strains that were re-ribotyped showed identical patterns (Figure 4.2). The patterns were compared with those of one reference 027 strain obtained from *C. difficile* Ribotyping Network for England and Northern Ireland reference laboratory at Leeds and were verified as the correct patterns.
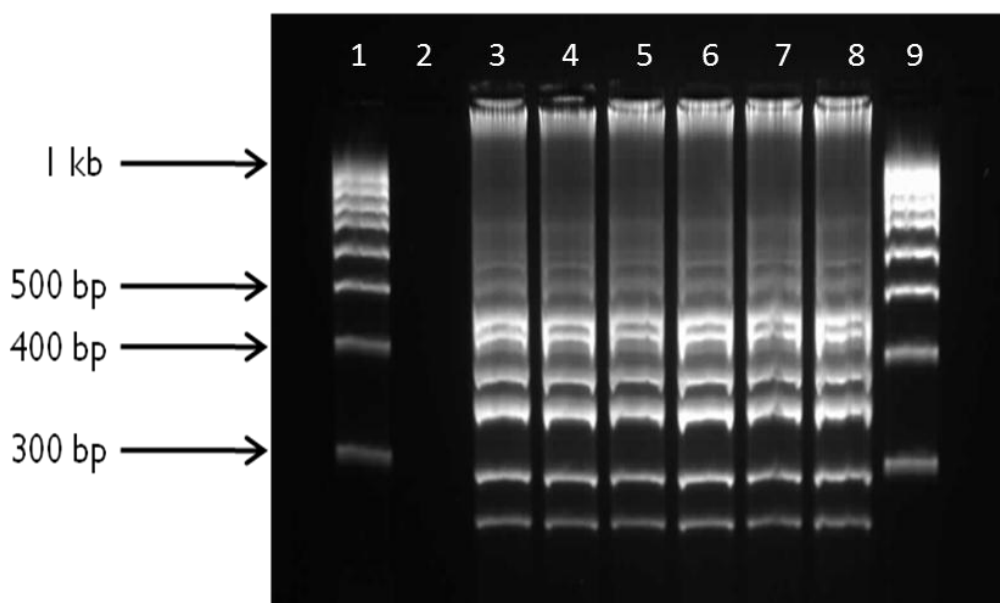
Figure 4.2 Agarose gel pictures showing PCR ribotype patterns of six randomly selected *C. difficile* 027 isolates used in this study.

DNA of *C. difficile* 027 isolates were ribotyped using PCR (Stubbs *et al.*, 1999). The PCR products were resolved on an agarose gel and the ribotypes profiles were determined by visualising on agarose gels. Lanes 1 and 9, represents a 1 kb marker (Fermentas, York, United Kingdom). Lane 2, is blank and Lanes 3-8 are the ribotype profiles of six randomly selected isolates used in this study.

4.3.2 Primer design

4.3.2.1 The capsid gene primers

The four available *C. difficile* myovirus sequences used for the design of the degenerate primers targeting the major capsid gene were phiC2, CD630, phiCD119 and phi12. The multiple alignment sequence of the four capsid sequences is shown in Figure 4.3. The sequences were aligned using ClustalW and conserved regions for the forward and reverse primers were between 11-31 nucleotides and 346-365 nucleotides respectively (Figure 4.3). Regions with either nucleotide deletions or high AT contents were avoided. The estimated amplicon size for the capsid gene was calculated as 358 bp which covers approximately 40 % of the gene. The capsid primers have 20 nucleotides each and had a melting temperature of 48 ^{o}C. The GC contents for the forwards and reverse primers were 38 % and 45 % respectively (Table 4.1).

```
•    phiC2     ATGGCAG---CACTAAATTATGCAAAAGAATATTCAAATGTTTTAGC---ACAAGCATAT 54
•    CD630     ATGGCAG---CACTAAATTATGCAAAAGAATATTCAAATGTTTTAGC---ACAAGCATAT 54
•    Phi12     ATGGCAG---CACTAAATTATGCAAAGAATATTCAAATGTTTTAGC---ACAAGCATAT 54
•    CD119     ATGGCTAATACACTAGCGTACGGACAAGTTTTACAACAAGGATTGGATAAACAAGCA-AC 59
•              *****     *****   ** * *** *     * * ** *     ******* *
                          Forward →

•    phiC2     CCTTATACTTTAAACTTCGGGGATTTGTATGCAACACCAAATAA-TGGAAGATATAGATG 113
•    CD630     CCTTATACTTTAAACTTCGGGGATTTGTATGCAACACCAAATAA-TGGAAGATATAGATG 113
•    phi12     CCTTATACTTTAAACTTCGGGGATTTGTATGCAACACCAAATAA-TGGAAGATATAGATG 113
•    CD119     ACAAGAATTATTAACTGGTTGGATGGAT-TCTAATGCTAAACAAATAAAATATGAAGGAG 118
•              *    * * * ****   ****   * * ** * *** ** *  ** **  ** *

•    phiC2     GACTGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGAGTAGATTCAAA 173
•    CD630     GACTGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGAGTAGATTCAAA 173
•    phi12     GACTGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGAGTAGATTCAAA 173
•    CD119     GA--------AAAGAAGTAAAAATAGGTAAGCTTTCTACAGATGGTTTAGGAGATTATTC 170
•              **         *** * ** *****   *     * ****** ***    ** *****

•    phiC2     CAGAGATACAA-TAGCAGTA--GCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGT 230
•    CD630     CAGAGATACAA-TAGCAGTA--GCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGT 230
•    phi12     CAGAGATACAA-TAGCAGTA--GCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGT 230
•    CD119     AAGAGGTTCAGCTAATGCTTATGTTGGTGGAGATGTTAAATTTGAATATGAAACTAAAAC 230
•              **** * ** **      *   * *      ** *   * *     ** *  *** ****

•    phiC2     ATTAACT---AATCAAAGGAAATGGTCAACATTGGTTCATCCAGCAGATATAAACCAAAC 287
•    CD630     ATTAACT---AATCAAAGGAAATGGTCAACATTGGTTCATCCAGCAGATATAAACCAAAC 287
•    Phi12     ATTAACT---AATCAAAGGAAATGGTCTACATTGGTTCATCCAGCAGATATAAACCAAAC 287
•    CD119     AATGACTCAAGATAGAGGGAGAAAATTCACATTAG---ATGCTATGGATGTAGATGAAAC 287
•              * * ***     ** * *** *   *  ***** *   ** *  *** ** *  ****

•    phiC2     TAATTATGTGGCTTCAATAGGCAATATAACAAAAGTATATAATGAGGAACAAAAGTTTCC 347
•    CD630     TAATTATGTGGCTTCAATAGGCAATATAACAAAAGTATATAATGAGGAACAAAAGTTTCC 347
•    Phi12     TAATTATGTGGCTTCAATAGGCAATATAACAAAAGTATATAATGAGGAACAAAAGTTTCC 347
•    CD119     AAATTTCTTAGTAACAGCAACGACTGTCATGGGAGAATTTCAAAGGTTAAAAGTCATACC 347
•              ****   * *   ** *   * * * *   ** ** * *   * * **  *   * **←

•    phiC2     AGAGATGGATGCTTACTGTATATCTAAAATATATGCTGATTGGACCG-CATTAGGTAATA 406
•    CD630     AGAGATGGATGCTTACTGTATATCTAAAATATATGCTGATTGGACCG-CATTAGGTAATA 406
•    Phi12     AGAGATGGATGCTTACTGTATATCTAAAATATATGCTGATTGGACCG-CATTAGGTAACA 406
•    CD119     AGAGATAGATGCTTATAGATTAAGTCGTCTAGCTACTATTGCTATAGGTATAAAAGGAGA 407
•              ****** ********   *  **   **  * **   *   * ** *      * *
                   Reverse
```
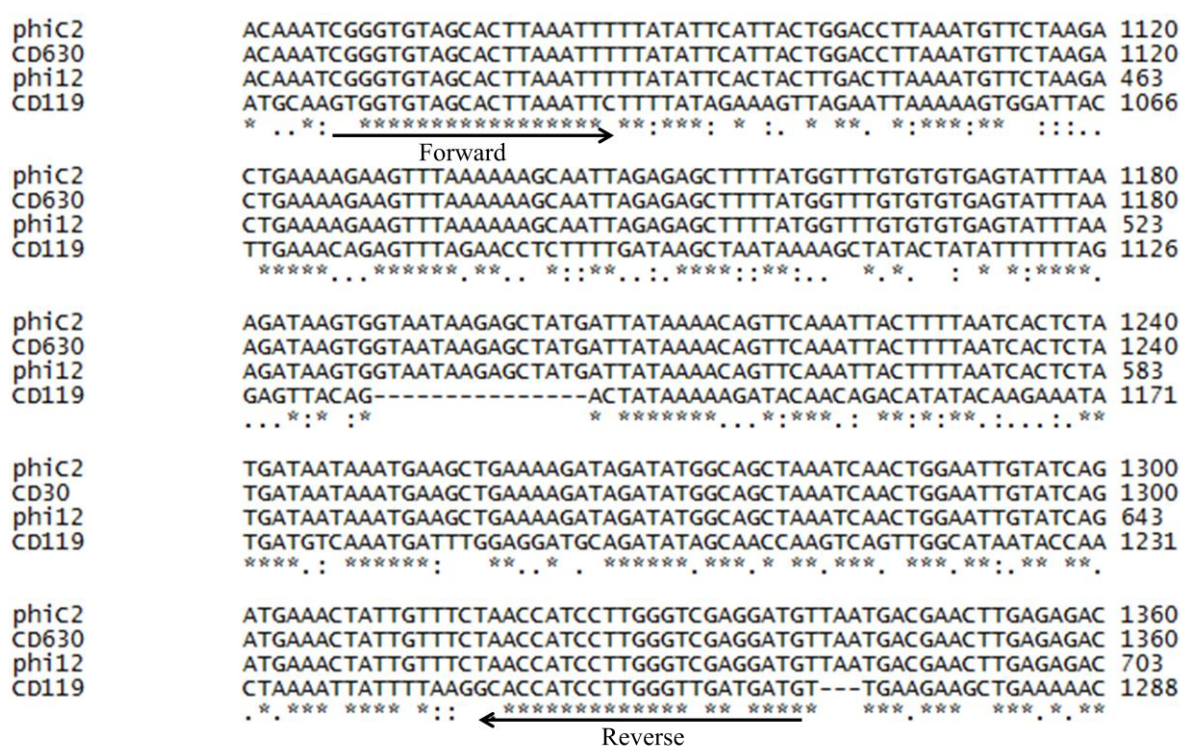
Figure 4.3 Multiple sequence alignment of four *C. difficile* phage sequences used in the design of the capsid primers.

DNA sequences of PhiC2, CD630 and phiCD119 were obtained from NCBI searches and phi12 was a partial DNA sequences provided by Katherine Hargreaves, University of Leicester. The sequences were aligned using ClustalW. Identical nucleotides are shown using asterisks. Regions for the forward and reverse primers were manually selected and indicated by arrows.

Table 4.1 Oligonucleotides primers used in this study

Gene	Sequence	MgCl$_2$ concentration	Annealing temperature	Amplicon size	Source
Capsid	5'- CAC TAR MKT AYG SAM AAG WW-3' 5'- CWR TAA GCA TCY ATC TCT GG -3'	3 mM	48 °C	358 bp	This project
Portal	5'- SKG GTG TAG CAC TTA AAT TY-3' 5'- CAT CMT CRA CCC AAG GAT GG-3'	4 mM	44 °C	273 bp	This project
Holin	5'- TAT ACC AGA GCA GTT RCT RA-3' 5'- CMT CCT TCA AYT GTT TGT AA-3'	3 mM	48 °C	227 bp	Dr Jinyu (University of Leicester)

The above table shows the sequences of the capsid, portal and the holin protein gene primers used in this study. The PCR conditions for the primers were optimised by adjusting the MgCl$_2$ concentrations and annealing temperatures. The optimised PCR conditions, amplicon sizes and sources of the primers are also shown in the table.

4.3.2.2 The portal gene primers

The same four candidate phage sequences were used to design the portal protein gene primer using the same method described in section 4.3.2.1 above. The 273 bp region selected for the forward and reverse primers was between 1013-1032 nucleotides and 1251-1270 nucleotides respectively (Figure 4.4). This bears approximately 19 % of the gene. The forward and the reverse primers for the portal gene also consist of 20 nucleotides and had a GC content of 40 %. Melting temperature for these primers was 44 ^{o}C (Table 4.1).

```
phiC2   ACAAATCGGGTGTAGCACTTAAATTTTTATATTCATTACTGGACCTTAAATGTTCTAAGA 1120
CD630   ACAAATCGGGTGTAGCACTTAAATTTTTATATTCATTACTGGACCTTAAATGTTCTAAGA 1120
phi12   ACAAATCGGGTGTAGCACTTAAATTTTTATATTCACTACTTGACTTAAAATGTTCTAAGA 463
CD119   ATGCAAGTGGTGTAGCACTTAAATTCTTTTATAGAAAGTTAGAATTAAAAAGTGGATTAC 1066
        *  ..*:  *****************:  **:***:  *  :.  *  **.  *:***:**    :::..
                              _____>
                                    Forward
phiC2   CTGAAAAGAAGTTTAAAAAAGCAATTAGAGAGCTTTTATGGTTTGTGTGTGAGTATTTAA 1180
CD630   CTGAAAAGAAGTTTAAAAAAGCAATTAGAGAGCTTTTATGGTTTGTGTGTGAGTATTTAA 1180
phi12   CTGAAAAGAAGTTTAAAAAAGCAATTAGAGAGCTTTTATGGTTTGTGTGTGAGTATTTAA 523
CD119   TTGAAACAGAGTTTAGAACCTCTTTTGATAAGCTAATAAAAGCTATACTATATTTTTTAG 1126
        *****...  ******.**..  *::**..:.****::**:..  *.*.  :  *  *:****.

phiC2   AGATAAGTGGTAATAAGAGCTATGATTATAAAACAGTTCAAATTACTTTTAATCACTCTA 1240
CD630   AGATAAGTGGTAATAAGAGCTATGATTATAAAACAGTTCAAATTACTTTTAATCACTCTA 1240
phi12   AGATAAGTGGTAATAAGAGCTATGATTATAAAACAGTTCAAATTACTTTTAATCACTCTA 583
CD119   GAGTTACAG--------------ACTATAAAAAGATACAACAGACATATACAAGAAATA 1171
        ...*:* :*          *  *******...*:***.:  **:*:**.:...:.**

phiC2   TGATAATAAATGAAGCTGAAAAGATAGATATGGCAGCTAAATCAACTGGAATTGTATCAG 1300
CD30    TGATAATAAATGAAGCTGAAAAGATAGATATGGCAGCTAAATCAACTGGAATTGTATCAG 1300
phi12   TGATAATAAATGAAGCTGAAAAGATAGATATGGCAGCTAAATCAACTGGAATTGTATCAG 643
CD119   TGATGTCAAATGATTTGGAGGATGCAGATATAGCAACCAAGTCAGTTGGCATAATACCAA 1231
        ****.:  ******:    **..*  .  ******.***.*  **.***.  ***.**:.** **.

phiC2   ATGAAACTATTGTTTCTAACCATCCTTGGGTCGAGGATGTTAATGACGAACTTGAGAGAC 1360
CD630   ATGAAACTATTGTTTCTAACCATCCTTGGGTCGAGGATGTTAATGACGAACTTGAGAGAC 1360
phi12   ATGAAACTATTGTTTCTAACCATCCTTGGGTCGAGGATGTTAATGACGAACTTGAGAGAC 703
CD119   CTAAAATTATTTTAAGGCACCATCCTTGGGTTGATGATGT---TGAAGAAGCTGAAAAAC 1288
        .*.***  ****  *::  <_____  **  *****    ***.***  ***.*.**
                              Reverse
```

Figure 4.4 Multiple sequence alignment of four *C. difficile* phage sequences used in the design of the portal gene primers.

DNA sequences of PhiC2, CD630 and phiCD119 were obtained from NCBI searches. Phi12 was a partial DNA sequence of a *C. difficile* phage from our laboratory. The sequences were aligned using ClustalW. Regions for the forward and reverse primers were manually selected and indicated by arrows.

4.3.2.3 The holin gene primers

The holin primers were designed and kindly donated by Dr Jinyu Shan (Shan *et al.*, 2012). All primers consist of 20 nucleotides (Table 4.1). The primers had a melting temperature of 48 $^{\circ}$C and a GC content of 40 %. The expected amplicon size is 227 bp which covers 88 % of the gene.

4.3.3 PCR amplification

4.3.3.1 *In silico* PCR

Before setting up the traditional PCR, all the primer sets were amplified the 42 available *Clostridium sp* genomes using online *in silico* PCR software. Within the available *Clostridium sp* genomes, six (CD2007855, CD630, CD196, CF5, M120 and R20291) were *C. difficile* strains. Three (CD2007855, CD196 and R20291) of the *C. difficile* genomes were of ribotype 027 (Stabler *et al.*, 2008, Stabler *et al.*, 2009), strain CD630 was ribotype 012, CF5 was ribotype 017 and M120 was ribotype 078 (Sebaihia *et al.*, 2006, Bordeleau *et al.*, 2011).

The *in silico* PCR amplification confirmed that all the primers were specific to *C. difficile* only. Among the six *C. difficile* genomes sequences available in the on-line *in silico* PCR software, the capsid primers matched sequences from *C. difficile* CD2007855, CD630, CD196, CF5 and R20291, and produced a predicted product of the expected size (Figure 4.5). It was also observed that the capsid primers were unable to amplify the genome of *C. difficile* M120 (Figure 4.5). The same amplification profile was observed with the holin gene primers. The expected product of 227 bp was produced with all the available *C. difficile* genomes except *C. difficile* M120 (Figure 4.6). However, using the portal gene primers, a single product of correct molecular weight of 273 bp was produced with *C. difficile* CD630 and CF5 only. No product was

observed with any of the 027 ribotype genomes (CD2007855, CD196 and R20291) and

with *C. difficile* M120 which is of ribotype 078 with the portal primers (Figure 4.7).
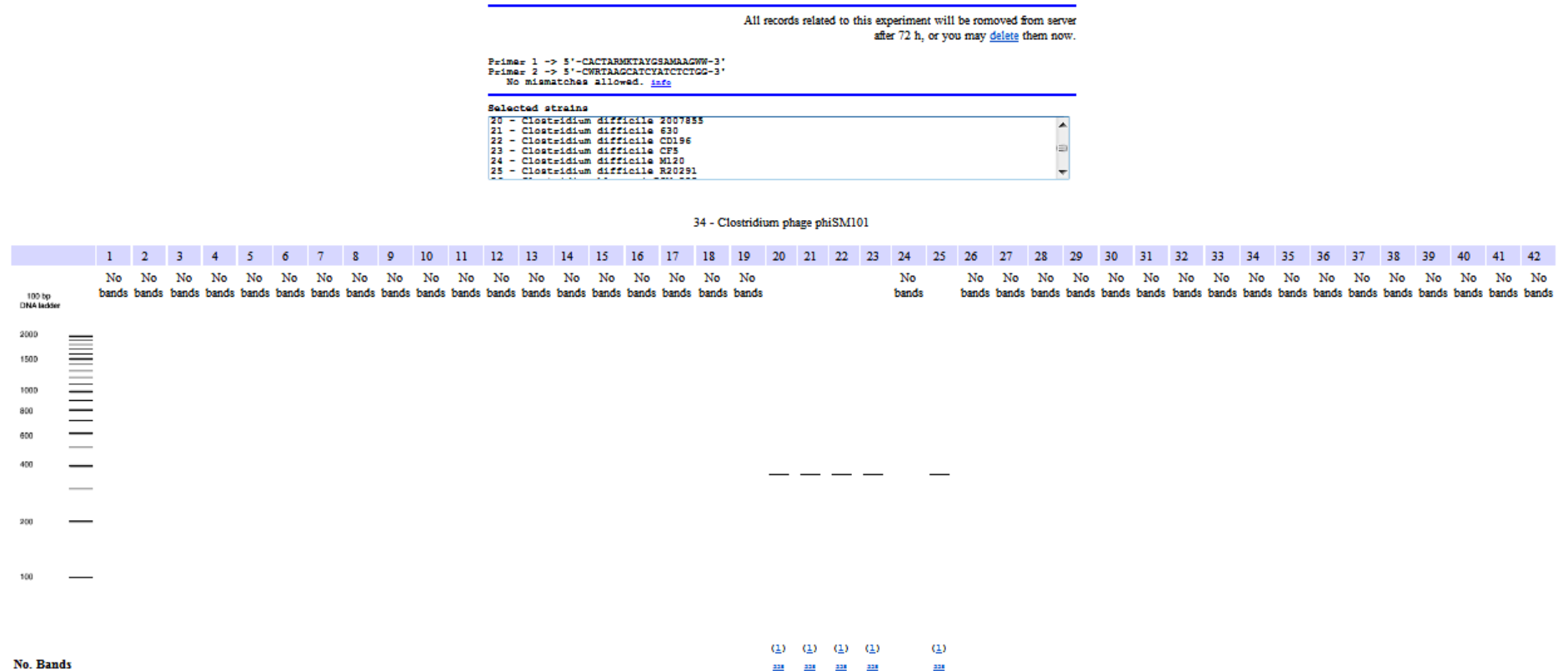
Figure 4.5 Picture showing *in silico* PCR amplification of the capsid gene primers.

In silico PCR amplification using the capsid primers is shown above. A PCR product with a molecular weight of 358 bp was obtained with *C. difficile* CD630, CD2007855, CD196 and R20291. No band was observed with M120 using the capsid primers with the *in silico* PCR. Lanes 1-42 represent the available genomes of *Clostridium sp* in the online *in silico* PCR. These consist of the following:

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1 - *Clostridium acetobutylicum* ATCC824

2 - *Clostridium acetobutylicum* DSM 1731

3 - *Clostridium acetobutylicum* EA 2018

4 - *Clostridium acidurici* 9a

5 - *Clostridium beijerinckii* NCIMB 8052

6 - *Clostridium botulinum* A str. ATCC 19397

7 - *Clostridium botulinum* A str. ATCC 3502

8 - *Clostridium botulinum* A str. Hall

9 - *Clostridium botulinum* A2 str. Kyoto

10 - *Clostridium botulinum* A3 str. Loch Maree

11 - *Clostridium botulinum* B str. Eklund 17B

12 - *Clostridium botulinum* B str. Eklund 17B(NRP)

13 - *Clostridium botulinum* B1 str. Okra

14 - *Clostridium botulinum* BKT015925

15 - *Clostridium botulinum* Ba4 str. 657

16 - *Clostridium botulinum* E3 str. Alaska E43

17 - *Clostridium botulinum* F str. 230613

18 - *Clostridium botulinum* F str. Langeland

19 - *Clostridium botulinum* H04402 065

20 - *Clostridium cellulolyticum* H10

21 - *Clostridium cellulovorans* 743B

22 - *Clostridium clariflavum* DSM 19732

23 - *Clostridium difficile* 2007855

24 - *Clostridium difficile* 630

25 - *Clostridium difficile* CD196

26 - *Clostridium difficile* CF5

27 - *Clostridium difficile* M120

28 - *Clostridium difficile* R20291

29 - *Clostridium kluyveri* DSM 555

30 - *Clostridium kluyveri* NBRC 12016

31 - *Clostridium lentocellum* DSM 5427

32 - *Clostridium ljungdahlii* ATCC 49587

33 - *Clostridium novyi* NT

34 - *Clostridium perfringens* NCTC 8237

35 - *Clostridium perfringens* ATCC 13124

36 - *Clostridium perfringens* SM101

37 - *Clostridium phage* phiSM101

38 - *Clostridium phytofermentans* ISDg

39 - *Clostridium saccharolyticum* WM1

40 - *Clostridium sp*. BNL1100

41 - *Clostridium sp*. SY8519

42 - *Clostridium sticklandii* DSM 519

Figure 4.6 Picture showing *in silico* PCR amplification of the holin gene primers.

Using the holin primers, an amplican size of 227 bp was obtained with *C. difficile* CD630, CD2007855, CD196 and R20291. No band was observed with *C. difficile* M120 using the holin primers in the *in silico* PCR. Lanes 1-42 represent the available genomes of *Clostridium sp* in the online *in silico* PCR as shown in Figure 4.6.
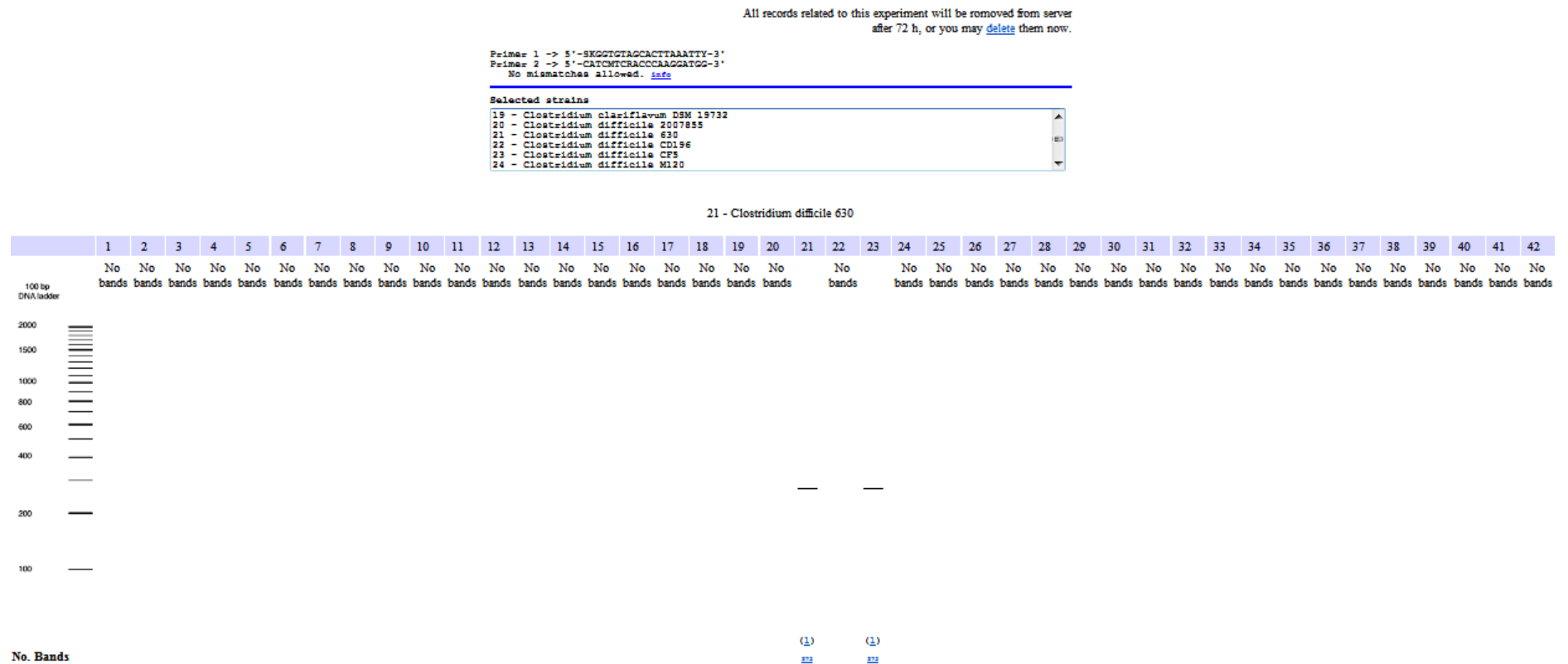
Figure 4.7 Picture showing *in silico* PCR amplification of the portal gene primers.

With the portal primers, *in silico* PCR produced a band of molecular weight of 273 bp with *C. difficile* CD630 and CF5. No band was observed with *C. difficile* CD2007855, CD196 and R20291 and M120 with the primers in the *in silico* PCR conditions. Lanes 1-42 represent the available genomes of *Clostridium sp* in the online *in silico* PCR as shown in Figure 4.6.

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4.3.3.2 Traditional PCR amplification

The optimised $MgCl_2$ concentrations and annealing temperatures for the three PCRs are shown in Table 4.1. The optimum $MgCl_2$ concentration was 3 mM for the capsid and holin primers and was 4 mM for the portal primers. The DNA template from phi12, a *C. difficile* myovirus was used for the optimisation of the PCR conditions. The primers were then used to amplify 12 phage DNA samples that could be induced at high abundance from the different *C. difficile* 027 MLVA and pulsovar types isolates used in this study. The 027 phage consisted of two phage tail-like particles (45L and 41L), three putative myoviruses (96L, 91L and 16L) and seven defective myoviruses (84L, 82L, 52L, 68L, 90L, 80L and 73L). In addition to being able to induce these phages at high abundance, the isolates were also selected to cover all the subtypes morphologies of the myoviruses induced from isolates examined in this study.

After the phage DNA was extracted, the PCR was set up for the phage genes. Since the phages were temperate (i.e. they integrate into their host genome), the primers were also used to amplify the genomic DNA of the *C. difficile* hosts of the phages. To compare between sequences of the 027 ribotype and other ribotypes of *C. difficile,* genomic DNA of nine additional *C. difficile* ribotypes (005, 001, 220, 015, 002, 012, 020, 014 and 078) were included in the PCR set up. A nuclease-free water sample, DNA samples of S-PM2 phage (a cyanophage), phiCD27 (*C. difficile* phage) and isolate 36L (which contained no inducible prophage) were included as negative controls. Two positive controls included the DNA samples of phi12, and CD630.

PCR amplification using the capsid primers produced the expected amplicon of ~358 bp with all the *C. difficile* 027 phage samples including the putative myoviruses, phage tail-like particles and the defective myoviruses. The same amplicon size was also obtained with phi12, CD630 and ribotypes 014, 005, 002, 020, 015, and 001 (Figure

4.8). However, no product was obtained from representative DNA samples of two ribotypes (220 and 078) and the all the negative controls (nuclease free water, S-PM2, phiCD27 and 36L).
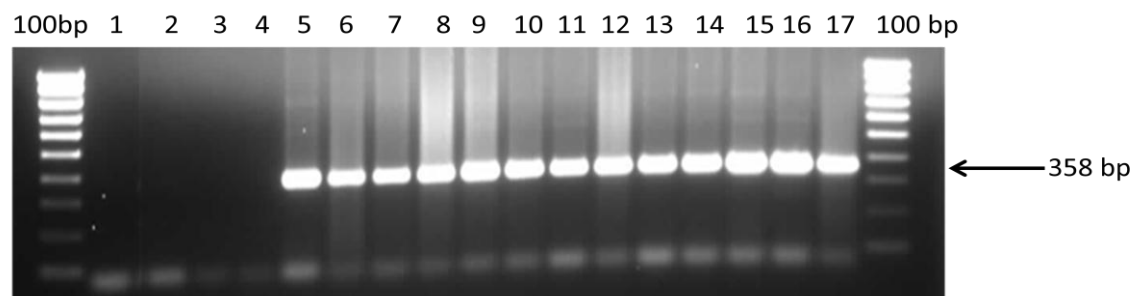
Figure 4.8 A gel picture showing 358 bp bands obtained from PCR amplification of the myovirus capsid gene.

Lanes 1 to 4 are negative controls (water, S-PM2, 36L and phiCD27 respectively). Lanes 5 and 6 are Phi12 and CD630 respectively. Lanes 7-17 are *C. difficile* 027 phages in this study. The 100-bp molecular standards (Fermentas, York, United Kingdom) are shown at the right and left corners of the gel.

With the holin primers, a PCR product of ~227 bp was obtained for all the DNA samples of putative myoviruses, phage tail-like particles and defective myoviruses induced from *C. difficile* isolates tested (Figure 4.9). In addition, the correct amplicon size was also obtained with phiCD27 and ribotypes 220 and 078 which were not amplifiable with the capsid primers. As expected, no band was produced with the negative controls (nuclease free water, S-PM2 and 36L) with the holin primers.
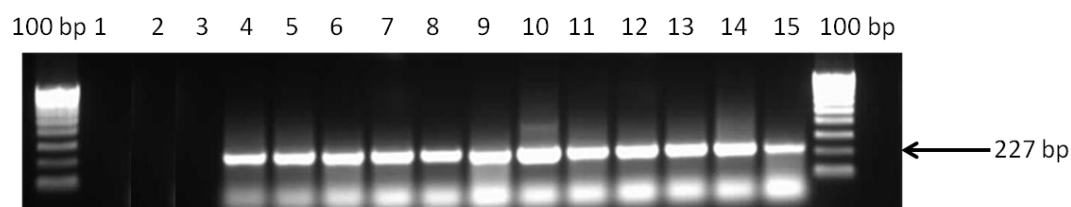
Figure 4.9 A gel picture showing 227 bp amplicons from PCR amplification of the phage myovirus holin gene

Lanes 1 to 3 are negative controls (water, S-PM2 and 36L respectively). Lanes 4, 5 and 6 are phiCD27, Phi12 and CD630 respectively. Lanes 7-15 are *C. difficile* 027 myoviruses induced in this study. The 100 bp markers are shown at the right and left corners of the gel.

The portal gene primers gave a PCR product of ~273 bp with all of the *C. difficile* 027 isolates tested despite the fact that none of the 027 strains were applified with these primers during the *in silico* PCR. Furthermore, ribotype isolates of 012, 001, 002, 015, 020, 078 and 014 were amplifiable with the portal primer. However, ribotype 005 did not produce a product under the optimum PCR conditions using the portal gene primers (Figure 4.10).
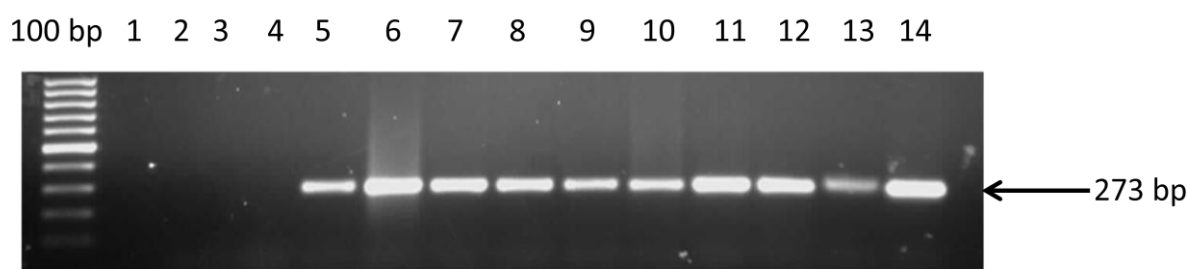
Figure 4.10 A gel picture showing 273 bp bands obtained after PCR amplification *C. difficile* myovirus portal gene

Lanes 1 to 3 are negative controls (water, S-PM2 and 36L respectively). Lane 4 is 005, Lanes 5 and 6 are Phi12 and CD630 respectively. Lanes 7-14 are *C. difficile* 027 phages induced in this study. The 100 bp markers are shown at the right and left corners of the gel.

In summary, both phage DNA and genomic DNA of the *C. difficile* 027 gave identical PCR results (Figure not shown). Only the holin primer set was able to amplify all the ribotypes used in this study. The capsid and the portal primers were unable to amplify three (220, 078 and CD27) and one (005) ribotype isolates respectively. Out of the ten ribotypes analysed, seven (027, 012, 014, 002, 020, 015 and 001) were amplifiable with all the primers.

4.3.4   Cloning of the PCR fragments and analysis of the plasmids

In order to produce multiple copies of the genes and sufficient DNA material for sequencing, the PCR amplified products were cloned. Therefore, the DNA products were gel purified, ligated to a vector and were then transformed into *E. coli* competent

cells. The transformed *E. coli* competent cells were analysed after 24 h culturing on LB

plates supplemented with 100 μl/ml ampicillin and 40 mg/ml X-gal. The blue and white

colonies were observed on the selective plates after 24 h incubation (Figure 4.11).
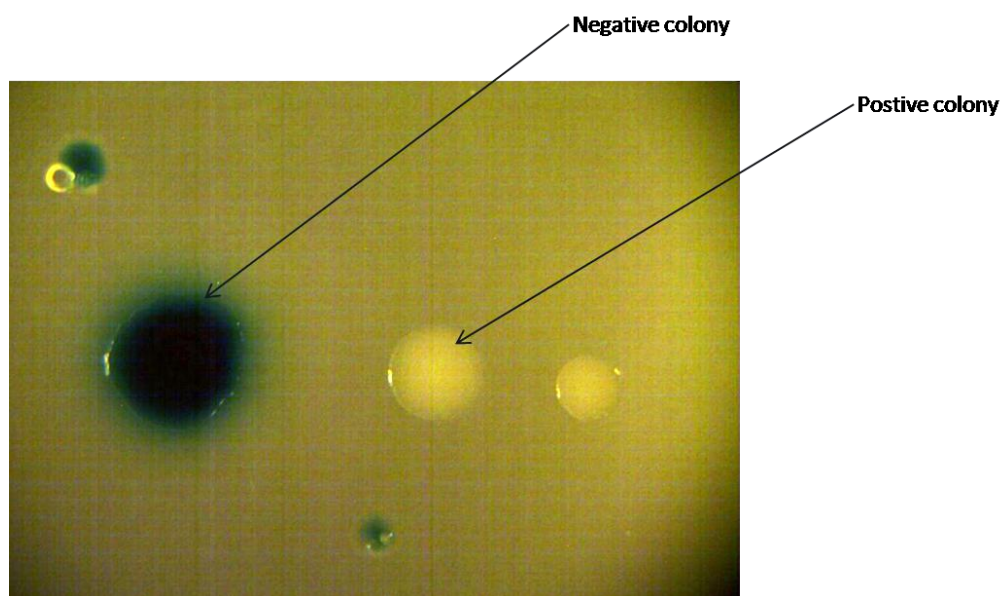
**Figure 4.11 Culture plate showing blue and white colonies of transformed
chemically competent *E. coli* cells growing on LB selective antibiotic medium.**

The white colonies contain the vectors with the ligated inserts while the blue colonies
indicate negative colonies which contain the vectors without the inserts.

After extracting plasmids from the prospective positive white colonies they were

digested with EcoR1. Resolution of the digested plasmid DNA on an agarose gel

showed an additional band (the insert) along with a ~3.9 kb band which is the vector

(Figure 4.12). Plamid DNA samples containing the inserts were sequenced by GATC

using the M13 forward primer 5'-TGTAAAACGACGGCCAGT-3'.
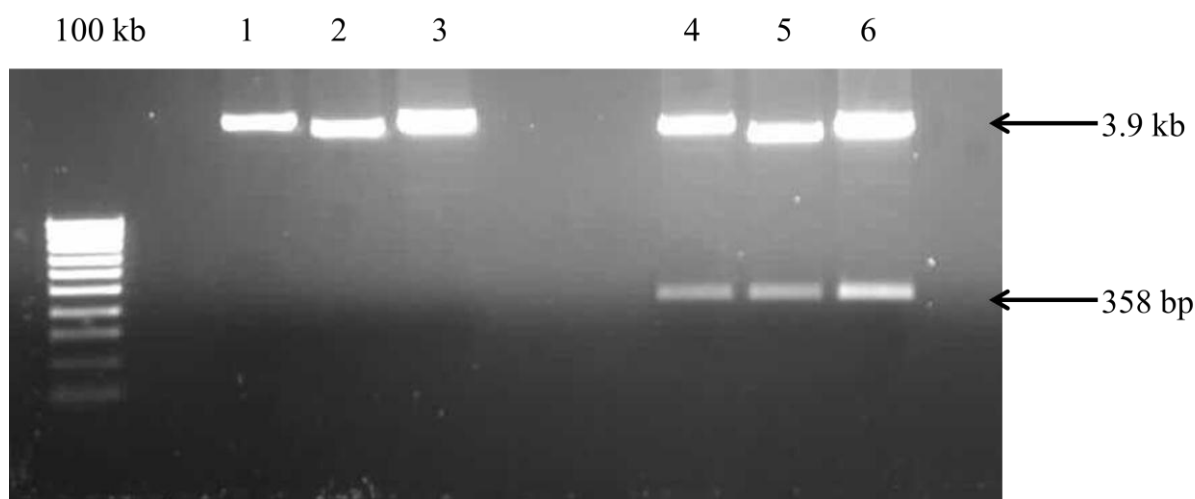
Figure 4.12 Gel picture showing DNA products obtained after digestion of the capsid plasmid using EcoR 1.

Lanes 1-3 are undigested plasmid DNA samples showing a molecular of ~ +3.9 kb. Lanes 4-6 are showing digested plasmid DNA. The larger molecular weight of the vector (about 3.9 kb) and the capsid insert ~ 358 kb can be seen on lanes 4-6.

The sequenced chromatogram files (.abi format) were downloaded from the GATC website and edited using Chromas Lite 2.01. To identify the insert within the plasmid sequences, five nucleotides CCCTT upstream and AAGGG downstream of the vector were each detected by using the Edit-Find command in the Chromas program (The upstream sequences are indicated by a black rectangular box in Figure 4.13). The excess nucleotide sequences which made up part of the plasmid were deleted. The Fasta format of the inserts sequences were copied using the Edit-Copy-sequences command. For the capsid and holin genes, sequences were confirmed to be 358 and 227 nucleotides respectively except 41L where the capsid sequence was 989 nucleotides (Appendix 4 and 5 respectively). The portal genes sequences were variable and ranged between 217 and 325 nucleotide (Appendix 6). Five isolates (ribotypes 001, 002, 014, 015 and 020) produced a sequence of 217 nucleotides with the portal primers. The portal gene sequence of isolates 96L, 91L, 73L, phiCD27 and CD630 had 273 nucleotides. Ribotype 023 and isolate 68L had a portal gene sequence of 310

nucleotides. Isolates 80L, 90L, 41L, 16L, 45L and 52L had 326 nucleotides (Appendix 6). All the capsid and holin sequences were truncated by 25 nucleotides (50 nucleotides for portal gene sequences) downstream and upstream to remove errors. The 25 truncated nucleotides upstream a capsid sequence are indicated by a red box in Figure 4.13.
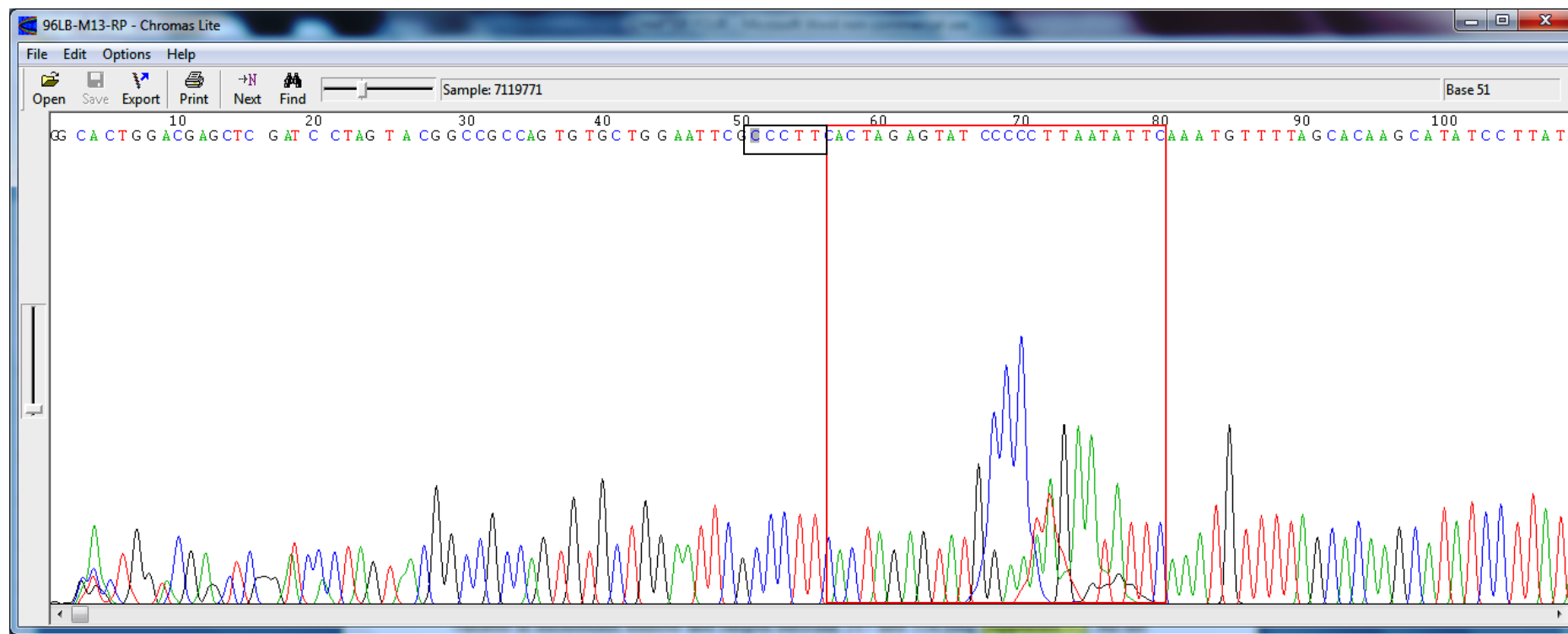
Figure 4.13 A chromatogram file of a sample 96L capsid sequence being edited using Chromas Lite 2.01.

The sample files were edited by first detecting the insert within the plasmid. This was done by detecting the five sequences upstream (CCCTT as shown in the black rectangular box) and five sequences AAGG downstream (not indicated in the figure because figure is too large) in the Edit-Find menu. Secondly, 25 nucleotides were deleted upstream (indicated in the red rectangular box) and downstream (downstream not shown).

Nucleotide BLAST (*blast*n) search confirmed that the sequences were correct. The sequences were then translated into amino acids using EBI-tool-sequence analysis-Transeq (Algorithm used was frame-6, table-Standard Code, regions- start-end, trim-No, Reverse-No and colour- No). One of the frames with no or minimum stop codons was chosen for further analysis. The amino acid sequences for the capsid, holin and portal genes are described in Appendixes 7-9. The translated proteins were again confirmed using the protein BLAST (*blast*p) search. Alignment of the amino acid sequences using ClustalX2 showed high homology among all the *C. difficile* 027 genes (Appendixes 7-1 to 7-11). The files (.phy files) generated from ClustalX2 were imported into Molecular Evolutionary Genetics Analysis (MEGA) package version 5.01 (MEGA5.01) and fully analysed (Tamura *et al.*, 2007, Kumar *et al.*, 2008). New alignment was built in MEGA5.01 and phylogenetic trees were generated using neighbour joining method with boostrap replication of 1000. Branches were proportionate to the length and the boostrap proportion of 75 % was considered to be identical.

4.3.5 Phylogenetic analysis of the sequences

4.3.5.1 The capsid protein

Phylogenetic analysis of the amino acid and nucleotide sequences of the capsid gene showed that *C. difficile* strains cluster together except *C. difficile* phiCD119 (Figure 4.14). Within this clade, all *C. difficile* 027 strains had identical sequences and therefore forming a single subclade (Subclade I) (Figure 4.14). Two other distinct subclades can be defined from the analysis of the capsid gene, subclade II and III. Subclade II is comprised of ribotypes 005, 002, 020 and 015 and Subclade III is comprised of *C. difficile* 001, *C. difficile* 012 (CD630) and *C. difficile* phage phiC2. Within Subclade III, *C. difficile* 012 (CD630) and phiC2 formed a tighter clade

(boostrap 99) (Goh *et al.*, 2007). The ribotype 014 sequence and phage phiCD119 are unrooted away from the other *C. difficile* ribotypes sequences. Regarding the outgroup taxa; the *E. coli* phage, *C. perfringens*, *Lactobacillus* and *Bacillus* clades are distinct from each other and from the *C. difficile* clade. However, *C. perfringens*, *Lactobacillus* and *Bacillus* isolates have identical sequences according to their genera and therefore form tight clades.
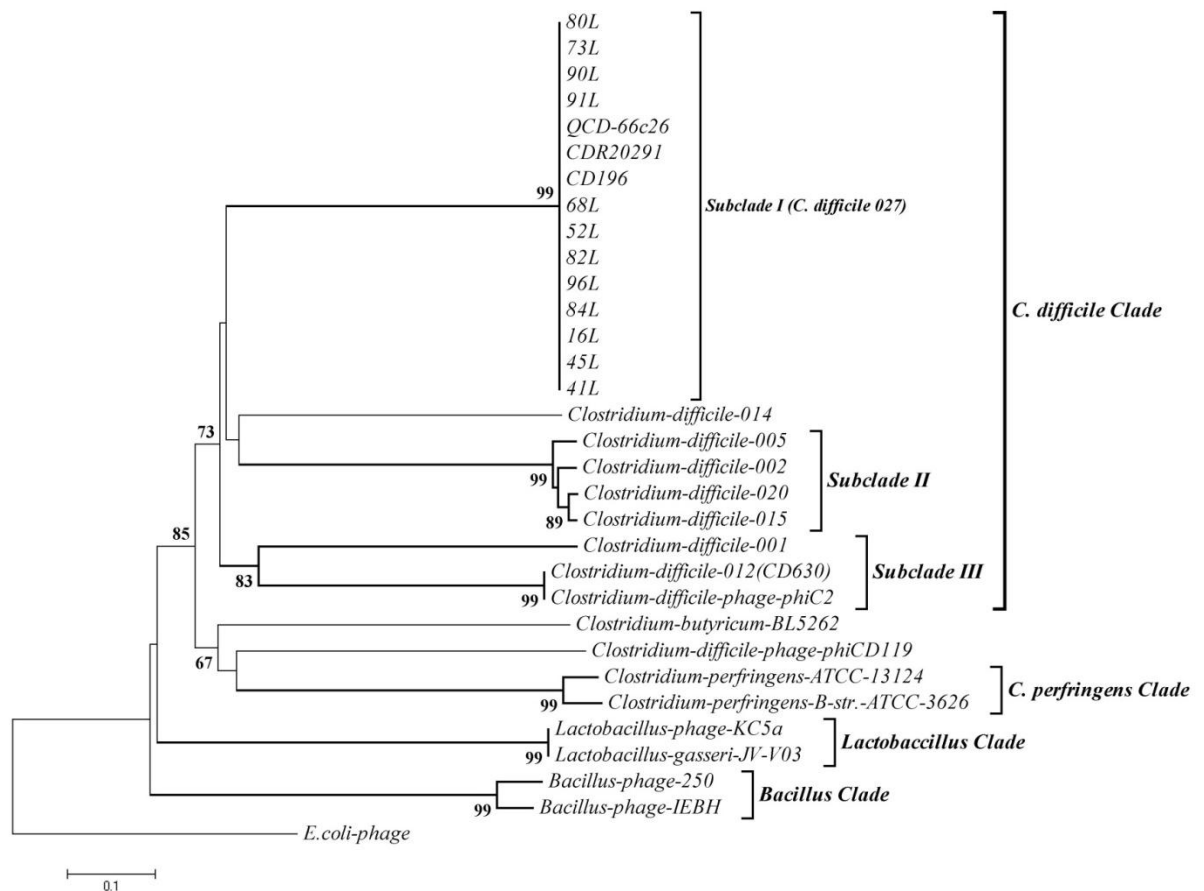
Figure 4.14 Evolutionary relationship of *Clostridium difficile* based on the myovirus capsid gene.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the *p*-distance method based on their amino acid sequences. All positions containing gaps and missing data were eliminated. The analysis involved 32 amino acid sequences of 12 (84L, 16L, 96L, 82L, 52L, 68L, 91L, 90L, 80L, 73L, 45L and 41L) representative isolates of the ribotype 027 subclades and six other ribotypes (ribotypes 014, 005, 002, 020, 015 and 001). Four other sequences including CD196, CDR20291 and QCD-66c26 (ribotype 027) and CD630 (ribotype 012) were obtained from *in silico* PCR. Other sequences were obtained from NCBI

searches. All sequences with 75 % similarities were assigned into a subclade. Evolutionary analyses were conducted in MEGA5.01

4.3.5.2 The holin protein

Phylogenetic analysis of the holin gene sequences produced two *C. difficile* clades, Clades I and II. The *C. difficile* Clade I is comprised of three subclades, Subclades I, II and III (Figure 4.15). The first subclade, Subclade I, is made up of all the *C. difficile* 027 isolates and *C. difficile* phage phiC2. Subclade II is comprised of *C. difficile* ribotypes 220 and 015 and the third subclade, is comprised of *C. difficile* ribotypes 020 and 014. Other *C. difficile* phage sequences, phiCD27 and phiCD119 and ribotypes 001, 002 and 012 fall outside the three subclades but within the *C. difficile* Clade I. The *C. difficile* Clade II had two isolates only, ribotypes 005 and 078. It is clear that the two (005 and 078) holin sequences are different considering their distance from the other *C. difficile* phages. The other outgroup taxa including *Bacillus sp, C. perfringens,* and *C. botulinum* clearly had identical holin sequences within their genera and formed distinct clade which is different from the *C. difficile* and *C. botulinum* clades and *E. coli* phage.
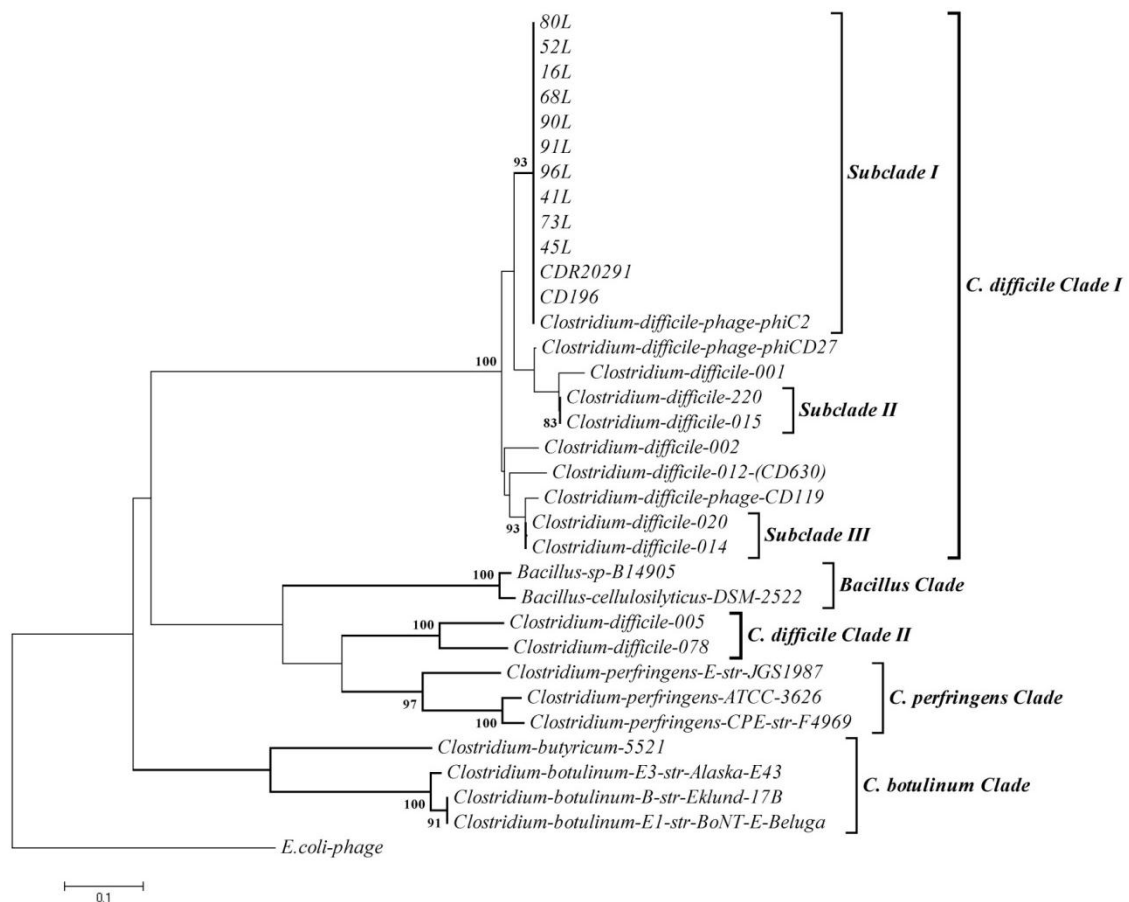
Figure 4.15 Evolutionary relationship of *Clostridium difficile* based on the myovirus holin gene.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the *p*-distance method based on their amino acid sequences. All positions containing gaps and missing data were eliminated. The analysis involved 34 amino acid sequences of 10 (80L, 52L, 16L, 68L, 90L, 91L, 96L, 41L, 73L and 45L) representative isolates of the ribotype 027 subclades and nine other ribotypes (ribotypes 001, 220, 015, 002, 012, 020, 014, 005 and 078). Four other sequences including CD196, CDR20291 and QCD-66c26 (ribotype 027) and CD630 (ribotype 012) were obtained from *in silico* PCR. Other sequences were obtained from NCBI searches. All sequences with 75 % similarities were assigned into a subclade. Evolutionary analyses were conducted in MEGA5.01

4.3.5.3 The portal protein

Just as the holin gene, there was no one defined *C. difficile* clade from the analysis of the portal gene. Phylogenetic analysis of the portal gene sequences yielded two *C. difficile* clades. The Clade I consist of all the 027 phages, *C. difficile* phage phiCD27 and five other ribotype isolates of 001, 002, 015, 020 and 078 (Figure 4.16). Within this clade, the ten *C. difficile* 027 phages from the induced 027 isolates as well as the two sequences (CD196 and CDR20291) formed a much tighter clade to form the *C. difficile* 027 Clade. Clade II comprised of *C. difficile* QCD-63q42, CF5 and ribotype 012 (CD630). Within the *C. difficile* Clade II, *C. difficile* M120 and CD630 formed a much tighter clade. Thus, the *C. difficile* ribotype 014 and *C. difficile* phages phiC2 and phiCD119 all fall outside the *C. difficile* Clade I and II. Similarly, the *C. perfringens* and *Bacillus sp* phage sequences did not cluster together according to their genera nor formed a common clade as observed in the capsid and holin gene trees.
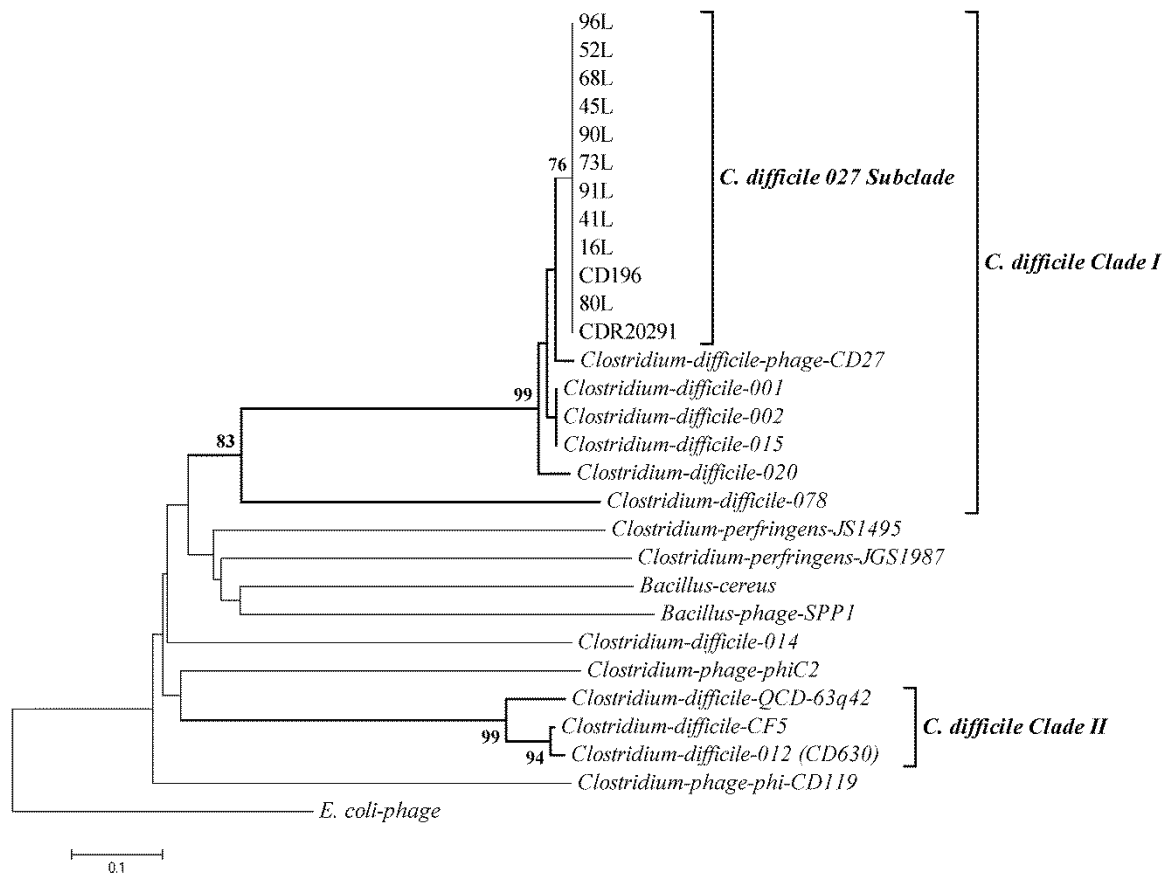
Figure 4.16 Evolutionary relationship of *Clostridium difficile* based on the myovirus portal gene.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the *p*-distance method based on their amino acid sequences. All positions containing gaps and missing data were eliminated. The analysis involved 29 amino acid sequences of 10 (96L, 52L, 68L, 45L, 90L, 73L, 91L, 41L and 16L) representative isolates of the ribotype 027 subclades and seven other ribotypes (ribotypes 001, 002, 015, 020, and 078). Two other sequences including ribotype 078 (M120) and ribotype 012 (CD630) were obtained from *in silico* PCR. Other sequences were obtained from NCBI searches. All sequences with 75 % similarities were assigned into a subclade. Evolutionary analyses were conducted in MEGA5.01

4.4 Discussion

The difficulties involved in the characterisation of C. *difficile* phages using methods that are culture dependent have motivated the work described in this section. Therefore this section focuses on the design of alternative methods for the identification and characterisation of these phages. Molecular markers to establish bacteriophage presence and diversity would be of clear interest in terms of unravelling C. *difficile* host-phage dynamics. Such information would greatly enhance morphological studies. In this study, three molecular markers were designed and tested on *C. difficile* 027 myoviruses which formed ~70 % (63/91) of the phages induced from strains involved in this study. The results obtained were also compared with nine other clinically relevant *C. difficile* ribotypes. The three primer sets targeted the conserved genes of *C. difficile* myoviruses including capsid, holin and the portal genes (Goh *et al.*, 2007).

The common occurrence of certain genes within viral genomes such as polymerase, terminase, portal and capsid proteins has led to the search of for a gene marker of viral diversity equivalent to the prokaryotic 16S rDNA gene (Sadeghifard *et al.*, 2006). Such gene can provide means to analyse the phylogenetic diversity within viral genomes. Unlike the prokaryotes, viruses lack a single universally recognised conserved gene or genetic marker such as the 16S rDNA that can be used for universal phylogenetic classification (Hendrix *et al.*, 1999, Roux *et al.*, 2011). Nevertheless, few relatively conserved genes that commonly occur within aquatic viral particles have been used to explore viral diversity (Marston and Sallee, 2003, Short and Suttle, 2005). For example, two structural genes identified in bacteriophage T4, the *gp20* and *gp23* encoding the capsid and the portal genes respectively have been used to study phylogenetic relationships of marine phage diversity (Hambly *et al.*, 2001, Zhong *et al.*, 2002, Dorigo *et al.*, 2004, Wang and Chen, 2004, Short and Suttle, 2005, Comeau and

Krisch, 2008). In a similar way, the phage DNA polymerase and the terminase genes have also been used to study diversity among viruses of the families *Myovirdae* and the *Siphoviridae* (Chen *et al.*, 1996, Casjens, 2008). Unfortunately, the DNA polymerase, gene could not be used for this purpose in *C. difficile* because this gene has not been detected in sequenced *C. difficile* phages (Goh *et al.*, 2007). In addition, the alignment of the terminase and integrase genes from *C. difficile* did not show nucleotide homology to identify conserved regions for suitable primer design. Thus, the primers designed for these genes were too degenerate to produce a PCR product. (Shan *et al.*, 2012). Therefore, three genes (capsid, holin and portal) were chosen and primers were designed for them.

Phylogenetic analysis of the genes was done both at the nucleotide and the amino acid level and both trees gave the same supported tree topology within *C. difficile* phages (Henn *et al.*, 2010). The amino acid sequences however allowed me to align the sequences from the *C. difficile* phages with phages which infect several other taxa and thus the analysis could be contextualised with respect to other phages present in related species.

It was observed that the capsid gene primer failed to amplify the DNA of M120, phage phiCD27 or ribotype 220. Reason for the inability of the capsid primer to amplify the genes of these phages may be attributed to the fact that phiCD27 has been reported to lack a capsid protein but possesses a head morphogenesis protein which is quite different (Mayer *et al.*, 2008). The *C. difficile* strain M120 and ribotype 220 isolates harbor prophages but also lack a recognisable capsid protein (Shan *et al.*, 2012). Similarly, the phylogenetic analysis of the capsid protein has classified phiCD119, a *C. difficile* myovirus, outside the *C. difficile* clade. This is possibly due to the absence of the major capsid protein but only the putative capsid protein in the genome of

phiCD119 (Goh *et al.*, 2007). The capsid DNA sequences of the phage tail-like particles show significant homology with the myovirus sequences, this report concurs with the previous study that these particles may share some evolutionary characteristics with the myoviruses (Nakayama *et al.*, 2000). Although, this marker has a potential to detect *C. difficile* myoviruses presence and may be useful in study of bacterial and viral species, it however may not be used as universal maker for *C. difficile* phages. This is because it cannot identify phages such as phiCD27, phiCD119, ribotype isolate of 220 and M120 which do not possess a recognisable capsid protein in their genome.

On the other hand, the holin gene had been identified in all the sequenced *C. difficile* phages, thus has an advantage over the capsid gene marker. Just like the capsid marker, the holin primer is able to amplify only genomes of *C. difficile* both with the *in silico* as well as the traditional PCRs. However, this primer failed to amplify the DNA of *C. difficile* M120 during the *in silico* PCR. Although this strain possesses a prophage in its genome (Henn *et al.*, 2010), the identified holin sequence is dissimilar to other *C. difficile* holin gene (Tan *et al.*, 2001). Protein BLAST (on 05/07/2012) using the holin amino acid sequence of M120 as a query showed no single hit for any *C. difficile* strains. The first five hits were *Acetivibrio cellulolyticus* CD2, *Clostridium cellulolyticum* H10, *Clostridium kluyveri* DSM 555, *Clostridium kluyveri* NBRC 12016 and *Thermoanaerobacter sp.* X514. Besides this very exceptional case, the holin marker has the ability to detect phages in the genome of all the ribotypes examined in this study and produced the desired amplicon size. Interestingly, phylogenetic analysis of the holin has also placed ribotypes 005 and 078 into a separate clade away from the rest of the *C. difficile* strains. This clearly shows that the holin sequences of these two ribotypes are different as previously reported (Shan *et al.*, 2012). This evidence further supports the fact that *C. difficile* M120 carry a different kind of holin gene, because

M120 is a ribotype 078 strain (Henn *et al.*, 2010). In addition, analysis of ribotyping dendograms generated from capillary gel electrophoresis, showed that ribotype 078 had low similarity to other ribotypes (Shan *et al.*, 2012). This could explain it position in the phylogenetic tree of the holin gene. In respect to its relationship to ribotype 005, the ribotype 078 showed only 25 % similarity using the ribotying dendogram but have 95 % similarity in the holin sequences (Shan *et al.*, 2012). Hence, it was suggested that there may be a horizontal gene transfer between these two strains (Shan *et al.*, 2012). However, the two strains did not contain the same morphology of phages. Ribotype 078 yielded a myovirus but only phage tail-like particles were induced from ribotype 005 upon induction with mitomycin C and norfloxacin. This may suggest that ribotype 005 may harbor other prophages in their genomes but are not inducible under the experimental conditions as suggested by Nale *et al.* (2012). All the 027 ribotypes showed holin gene homology with phiC2. Although the ribotype of the host strain of phiC2 is unknown, this phage has been shown to be closely related genotypically to a *C. difficile* 027 phage that was previously isolated (Fortier and Moineau, 2007, Goh *et al.*, 2007). It was observed that ribotypes 020 and 014 showed high similarity. Reason for this may be attributed to the fact that these two ribotypes are highly similar and may as well share a similarity in the holin gene (Marsden *et al.*, 2010).

Primer design for the portal protein was quite difficult as the alignment of the protein gene from the candidate phages showed very low regions of homology and there were a number of deletions in the gene sequences. It was therefore difficult to select a good region for the design of the primers. Therefore, the resultant primers possessed a high level of degeneracy and this may possibly explain why the *in silico* PCR could not amplify majority of the *C. difficile* DNA. In addition, the variable nucleotide number of the resultant amplicons form the traditional PCR may further

support the presence of deletions and insertions in this gene as observed in phiCD27 (Mayer *et al.*, 2008). The portal protein primer set, amplifies only a small region of this gene (19 %), thus its utility for subsequent phylogenetic analyses is limited due to insufficient coverage of the gene (Fuller *et al.*, 1998). Fuller *et al.* (1998) developed cynophage-specific primers to amplify the *gp20* gene which were able to identify 80 % of the cynanophages screened. However due to its small coverage, the primers failed to recognise 20 % of the cyanophage gene. In addition, the small coverage by the portal gene primer could explain its inability to amplify the three 027 strains available in the *in silico* PCR software. Therefore, as the sequence data of more *C. difficile* strains become available, new candidate strains can be included to allow better primer design to capture wider region of this gene. It was observed that only *C. difficile* CD630 and CF5 were amplifiable with the portal genes in the *in silico* PCR. This is because when the two genes were aligned, it was observed that *C. diffcile* CD630 and CF5 shared 99 % homology. BLAST search using CD630 as a query (10/07/2012) further supports the similarity of the two genes (Sebaihia *et al.*, 2006). In addition, the alignment of these genes with the 027 sequences showed that they share only about 20 % similarity. This further supports their position on the phylogenetic tree of the portal protein.

In order to gain insight into the molecular diversity of the genes (capsid, holin and portal genes) of *C. difficile* temperate myoviruses, a range of clinical isolates belonging to ten different ribotypes were examined. Among the 430 *C. difficile* ribotypes in the United Kingdom, only twenty ribotypes are recognised as clinically relevant (Warren Fawley, pers comm). In this study, ten *C. difficile* ribotypes have been examined to capture 50 % of the clinically relevant ribotypes in the United Kingdom. This will provide wide information of the heterogeneity of prophages contained in the genomes of these ribotypes.

Significant evidence suggests that myoviruses are the most common phages asscociated with *C. difficile.* All the ten ribotype isolates examined in this study have been shown to contain myoviruses and or phage tail-like particles in their genomes through prophage induction and TEM analysis (Nale *et al.*, 2012, Shan *et al.*, 2012). It is therefore clear that majority of the clinically relevant *C. difficile* ribotype harbor myoviruses in their genomes. (Goh *et al.*, 2005b, Fortier and Moineau, 2007, Goh *et al.*, 2007, Nale *et al.*, 2012, Shan *et al.*, 2012). In addition, some of the myoviruses have been reported to be associated with severe *C. difficile* disease or led to increased toxin production (Goh *et al.*, 2005a, Nale *et al.*, 2012).Therefore, the design of molecular makers to target *C. difficile* myoviruses will help detect phage presence in wide range of clinically relevant strains from different environmental sources without necessarily carrying out the cumbersome procedures of induction, isolation and TEM analysis. This early detection will greatly enhance patient management and direct treatment.

Although the myoviruses is the dominant phage morphology induced from *C. difficile*, there are diversities of subtypes within this morphology (Goh *et al.*, 2005b, Fortier and Moineau, 2007, Nale *et al.*, 2012, Shan *et al.*, 2012). Despite the differences in morphologies, the capsid, holin and the portal primers revealed that these genes are closely related within *C. difficile* and therefore forming a single clade except in the analysis of the holin and portal genes in which two *C. difficile* clades were identified. Although the *C. difficile* capsid, holin and portal sequences were closely related, it was observed that each of the ribotypes have unique gene sequences except in the holin sequences where the 027 isolates formed a tight clade with phiC2. However, the ribotype of phiC2 host is unknown. This report shows that there is a clear relationship between phage sequences and the ribotypes which reveals that the genetic diversity of *C. difficile* is reflected in the ribotyping scheme (Henn *et al.*, 2010). The observed

homologies in these genes within the *C. difficile* clade is in agreement with other previous reports (Goh *et al.*, 2007). The *C. difficile* phage clade is distinct from other members of the Clostridial family, and other bacterial species, which suggests that these phages have a narrow host range (Mayer *et al.* 2008). This possibly explains why it is difficult to identify susceptible hosts for the propagation of these phages in plaque assays and spot tests as observed in this study.

The ability of all the 027 sequences to form a strong grouping both at the nucleotide and amino acid level showed the much conserved nature of the genes of these ribotype (Henn *et al.*, 2010). This result is consistent with a previous report where DNA microarrays and Bayesian-based algorithms were used to phylogenetically analyse whole genomes of 75 *C. difficile* strains (Stabler *et al.*, 2006). Stabler *et al.* (2006) observed that the nineteen 027 strains studied appeared to form a tight group which was distinct from the other 56 strains. The observation that the 027 ribotype formed a tight group is also in agreement with previous work in which it was found that *C. difficile* 027 strains have very conserved genes, are descended from a single ancestor and acquire diverse external genetic element such as prophages, plasmids, antibiotic resistant genes and mutations via horizontal gene transfer during evolution (Henn *et al.*, 2010). These factors are likely to contribute to their pathogenicity (Sebaihia *et al.*, 2006).

4.5   Conclusion

Taken together, this data provide molecular insights into the genes of phages infecting *C. difficile* ribotypes. The analysis of the capsid, holin and portal gene sequences showed that the *C. difficile* 027 phages share a high level of gene homology. The relationship of these sequences with other ribotypes showed that they are unique but showed similarities at various levels as shown in the phylogenetic trees. Generally,

all the primers designed are specific to *C. difficile* myoviruses only. However, they have different abilities to detect phage presence due to the present or absence of specific recognisable proteins. The holin gene, though present in all *C. difficile* phages sequenced to date, have very different sequences that are not recognisable by these primers. The capsids primers showed good tree resolution but failed to identify phages that do not possess the major capsid protein such as phage phiCD27. The portal protein marker had high level of degeneracy and covered only a small portion of the gene which makes it unsuitable for *C. difficile* phylogenetic studies. These primer sets may be used singly or in combination to identify phage presence in *C. difficile* strains which will greatly aid morphological studies and strain choice before the time consuming and daunting process of prophage induction and TEM analysis. These molecular markers can be used to study phylogenetic relationships of *C. difficile* and other bacteria as well.

4.6 Future work

This study focused on molecular markers for the *C. difficile* myoviruses only. However, as more *C. difficile* siphoviruses sequences are becoming available, future work should focus on the design of molecular markers for this group of phages. Molecular markers with the ability to target both myoviruses and siphoviruses will greatly aid identification *C. difficile* strains which carry dual morphologies of phages. Other conserved genes in *C. difficile* myoviruses can be targeted for the design of molecular markers for these phages.

5 Comparative sporulation rates among *C. difficile* 027 strains

5.1 Introduction

Clostridium difficile is a Gram-positive, spore forming anaerobic bacterium and is the leading cause of community and nosocomial diarrhea (McMullen *et al.*, 2004, Orange *et al.*, 2007, Mann *et al.*, 2011, Hensgens *et al.*, 2012a, Voelker, 2012). Infection due to this bacterium has become more severe, more difficult to treat due to higher relapse and increased antibiotic resistance (McFarland *et al.*, 2007, Ananthakrishnan *et al.*, 2008, Sun *et al.*, 2011, Wells, 2011, Hensgens *et al.*, 2012b). The factors stated above have contributed significantly to the economic and clinical burden of CDI (Wilcox *et al.*, 1996, Dubberke *et al.*, 2008, Wang and Stewart, 2011, Wiegand *et al.*, 2012).

C. difficile infection is transmitted via the fecal oral route. Spores normally shed in the feces are able to withstand a variety of cleaning agents and harsh environmental conditions and can reside on hospital surfaces for prolonged period of time (Riggs *et al.*, 2007, Barbut *et al.*, 2009). One study has shown that *C. difficile* spores have the ability to persist up to 5 months on the surfaces of objects (Kim *et al.*, 1981). Therefore, the *C. difficile* spores can serve as reservoirs in hospital and community environments and play an important role in the transmission of *C. difficile* (Riggs *et al.*, 2007, Dumford *et al.*, 2009, Heeg *et al.*, 2012).

5.1.1 Morphological changes during spore formation

Generally, nutrient deprivation is recognised as the major factor that triggers bacterial endospore formation in bacteria (Labbe, 2005). Typically, seven distinct

morphological stages involved in sporulation are recognised in *Bacillus sp* (Figure 5.1). The stages are similar in *Clostridium sp* and are given below.

Stage I. Axial filament formation. In this stage the chromosome appears to change from a compact shape to a single rope or axial thread. The chromosome later segregate and divide into two (Figure 5.1) (Piggot and Losick, 2002).

Stage II. This stage is characterised by the formation of an asymmetrical double membrane between the two segregated chromosomes with little or no intervening peptidoglycan. The larger compartment is designated the mother cell, the smaller, the forespore (Figure 5.1) (Labbe, 2005).

Stage III. Engulfment of the forespore (Figure 5.1). The membrane formed in the larger compartment in Stage II begins to grow and surrounds the smaller compartment. The encircling edges from the larger compartment fuse to form the forespore and the cell is demarcated into two sections (the forespore and the mother cell) with opposite polarity (Woods and Jones, 1987). The segregated chromosome is packed in the forespore and degraded in the mother cell (Mohr *et al.*, 1991).

Stage IV. In this stage, the spore cortex between the two membranes is formed. Due to the engulfment process, the forespore is composed of two cyplasmic membranes, the inner and the outer membranes. In addition, there are two layers of peptidoglycan (the cortex and the germ cell walls) formed between the two layers. The cortex is composed of modified vegetative cell peptidoglycan and is consist of repeating subunits of muramic lactam subunit, alanine subunit and a tetrapeptide subunit (Figure 5.1) (Driks and Setlow, 2000).

Stage V. Appearance of coat protein. During this stage, the cystine-rich coat appears as discontinuous mass of coat material which link together to form a continuous

layer also known as the exosporium as shown in Figure 5.1. This coat material is exclusively made by the spore mother cell during sporulation (Driks, 2002).

Stage VI. This stage is characterised by the maturation of the cortex and coat components. The spore is composed of dipicolic acid which removes the spore divalent cations and the 3-phosphoglycerate, which serves as an energy source during germination (Driks and Setlow, 2000). The spore nucleoid is condensed into an A-helix state by the small acid-soluble spore proteins (SASPs) and serves as a nutrient source for the germinating spore (Driks, 2002). In Clostridia, the spore cell becomes swollen and forms a cigar-like structure with phase contrast microscopy and granulose accumulation (Figure 5.1) (Mohr *et al.*, 1991, Dürre and Hollergschwandner, 2004).

Stage VII. The spore is detached from the mother cell by a lytic enzyme. The anatomy of the spore is made of the following:

     a. an exosporium outer covering,

     b. a spore coat which is composed of several protein layers and is impermeable to toxins and chemicals,

     c. a cortex which occupies most of the spore volume and is made of unique less cross-linked peptidoglycan,

     d. a spore cell wall or core wall which surrounds the protoplast or core and

     e. the core protoplast which is metabolically inactive but is rich in DNA, ribosomes and DNA repair enzymes. The core is dehydrated, thus, having high resistance to dehydration and heat (Figure 5.1).
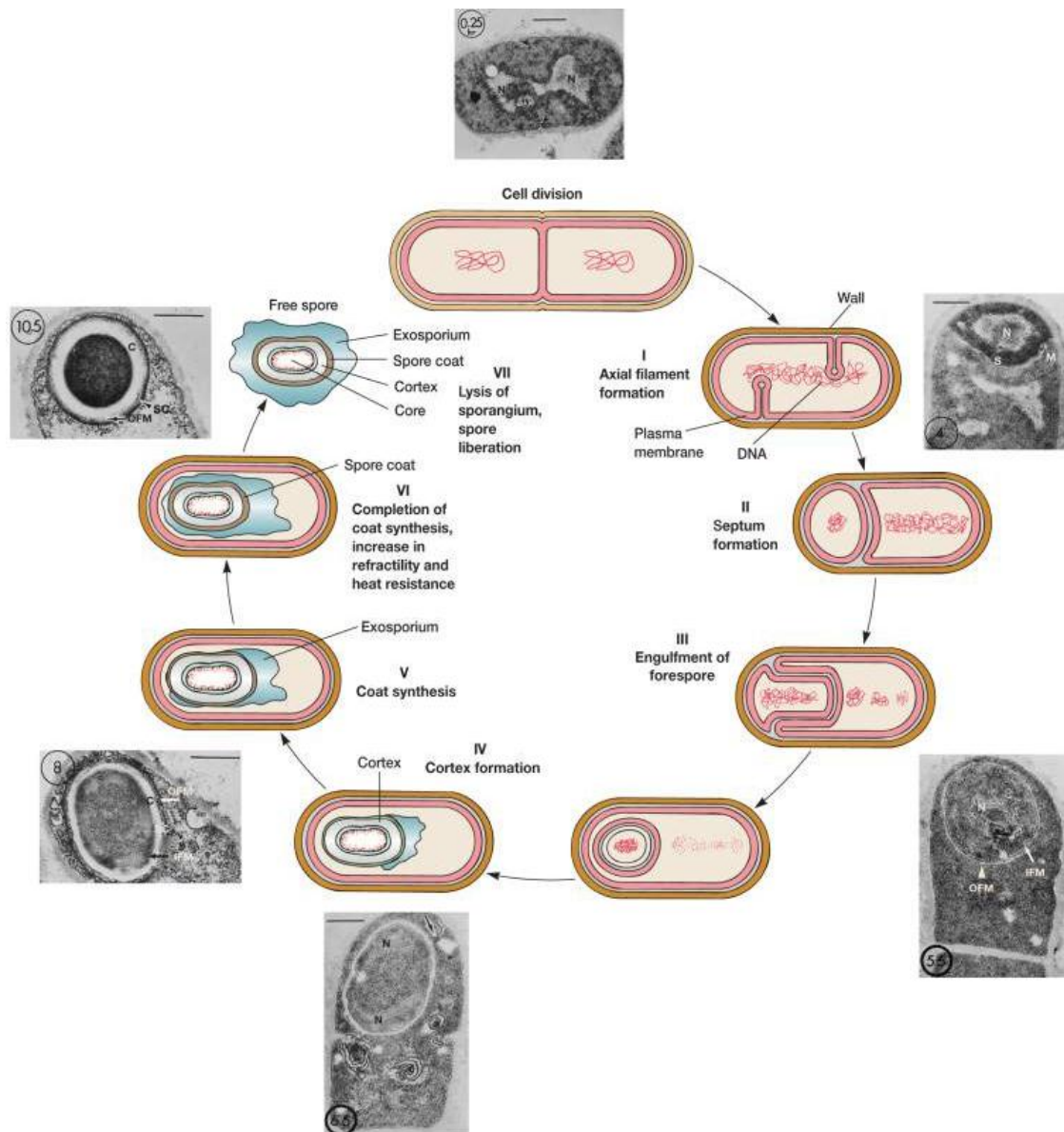
Figure 5.1 The various stages involved in the transformation of a bacterial vegetative cell into a spore.

Sporulation begins with axial chromosome formation (stage I), followed by formation of an asymmetrical double membrane (stage II) and engulfment of the forespore (stage III). Next is the formation of the spore cortex (stage IV) and coat (Stage V). The endospore matures (Stage VI) and become detached from the mother cell (stage VII). http://classroom.sdmesa.edu/eschmid/Lecture3-Microbio.htm.

5.1.2   Signal transduction and initiation of transduction during sporulation

The signal transduction pathway that controls sporulation initiation is extensively studied in Bacillus species. It is composed of a two-component signal transduction system (TCS) known as the sporulation phosphorelay (Burbulys *et al.*, 1991, Underwood *et al.*, 2009). External signals such as nutrient deficiency are recognised and transmitted via the histidine kinase which results in the autophosphorylation of a specific histidine (KinA or KinB) in the catalytic domain. The phosphate is transferred to the master regulator of sporulation, Spo0A via two other proteins, Spo0F and Spo0B. The first transmitter, Spo0F becomes phosphorylated by one of two histidine kinases, KinA and KinB at an aspartate residue. The phosphate group is then transferred to Spo0B which is subsequently transmitted to Spo0A. Phosphorylation of the active site aspartate of Spo0A promotes conformation of the adjacent output domain and thus, allows the proteins to bind to specific DNA sequences and to regulate transcription (Durre, 2005b).

In Clostridia, the mechanism of the phosphorelay doeas not exist (with exception of the Spo0A) (Stragier, 2002, Dürre and Hollergschwandner, 2004). Evidence to support this was shown in the genome sequences of members of the Clostridial family. It was reported that neither *spo0F-* or *spo0B-* homologous genes nor putative genes with high similarity to these genes could be detected in the genomes of *C. difficile* and *C. acetobutylicum* (Stragier, 2002). However, plausible candidate kinases that might phosphorylate Spo0A have been reported in *C. acetobutylicum, C. perfringens* and *C. tetani* (Paredes *et al.,* 2005). These differences might reflect the different environmental conditions that trigger sporulation in the two genera. In *Bacillus sp*, nutrient deficiency triggers sporulation to enable it survive but *Clostridium sp* relies on sporulation to escape unfavourable conditions such as the accumulation of organic acids due to

fermentative process and decrease in internal and external pH (Brown *et al.*, 1994, Dürre *et al.*, 2002).

5.1.3 Sporulation rates in *C. difficile* strains

The *C. difficile* ribotype 027 exists in diverse subclades which have varied disease severities (Fawley *et al.*, 2008). The re-emergence of this ribotype has contributed significantly to high disease severities and relapse due to antibiotic resistance (Pepin *et al.*, 2004, van der Kooi *et al.*, 2008). Many factors have been reported to have contributed to the hypervirulence nature and success of this pathogen. The 027 ribotype strains have been reported to produce large amount of toxins due to the carriage of binary toxin gene as well as the deletions in the negative regulatory gene (Voth and Ballard, 2005, Carter *et al.*, 2007). In addition, diverse temperate bacteriophages have been induced from 027 isolates and found to correlate to the subtypes (Nale *et al.*, 2012). Thus, phages play a significant role to *C. difficile* diversity and may consequently contribute to pathogencity. Also, strains belonging to this ribotypes are associated with high sporulation rates (Wilcox and Fawley, 2000, Fawley *et al.*, 2007, Akerlund *et al.*, 2008, Merrigan *et al.*, 2010, Cheng *et al.*, 2011, Vohra and Poxton, 2011). Although other reports have suggested otherwise, many reports have shown that the 027 strains produce high amounts of toxins (Rupnik *et al.*, 2009, Burns *et al.*, 2010a, 2010b, Carroll and Bartlett, 2011, Dingle *et al.*, 2011).

The controversy in these reports lies in the sample size which has been suggested to be very small (Burns and Minton, 2011). To date, no report has compared the sporulation characteristic of the 027 subtype's isolates. This study therefore, compared spore initiation time, spore concentration and morphological characteristics produced by a large population of *C. difficile* 027 isolates belonging to diverse subclades. The sporulation characteristics of the isolates are discussed in relation to the disease

severities shown in their subclades. Furthermore, it was discovered in our laboratory that one of the *C. difficile* phages sequenced encoded genes for histidine kinase which influence sporulation (Kate Hargreaves Pers Comm). This provides an incentive to determine if there is any obvious correlation between phage carriage and sporulation rates of the isolates examined.

5.2 Aims of study

1. To determine the spore initiation time and concentration of 41 C. *difficile* 027 subtypes isolates.

2. To correlate sporulation to the different MLVA and Pulsovar types

3. To correlate sporulation to the prophage contents of the isolates

5.3 Results

5.3.1 Spore formation rate of *C. difficile* 027 strains

The sporulation rates of 41 selected *C. difficile* 027 subtype isolates were determined and compared. The isolates are among the 91 *C. difficile* 027 isolates obtained from nine hospitals in England and have previously been reported to be categorised into 23 MLVA and 5 Pulsoavr subtypes (Fawley *et al.*, 2008). Among the 41 isolates examined, 40 (consisting of 14 MLVA and 3 Pulsovar subtypes) were obtained from patients in Hospital 1. Hospital 1 was selected due to its high number of isolates as well as great diversities of subtypes. One other isolate (36L, from MLVA 22, Pulsovar IV) obtained from a patient in Hospital 4 was also included in this study (Fawley *et al.*, 2008). Isolate 36L was included because it was found to yield no phage after inductions with mitomycin C and norfloxacin followed by TEM analysis (Nale *et al.*, 2012). The inclusion of this isolate will allow comparison of sporulation rates between prophage containing isolates and the non-prophage containing one. Both Hospitals 1 and 2 belong to the Institution A, which was among the four Institutions previously reported to have the highest number of early deaths in the UK (29 % deaths within the first 30 days) (Fawley *et al.*, 2008). The isolates identities and their corresponding MLVA and Pulsovar subtypes are shown in Table 5.1 and Table 5.2 respectively.

Table 5.1 Summary of the 40 *C. difficile* 027 MLVA subtype isolates used in this study

MLVA type	No of isolates	Isolate IDs
1	3	14L, 52L, 38L
2	1	2L
5	1	32L
6	2	17, 81L
7	6	67L, 61L, 54L, 75L, 77L, 21L
8	1	5L
12	1	63L
14	2	23L, 47L
15	1	59L
16	13	3L, 55L, 1L, 42L, 15L, 16L, 19L, 30L, 35L, 56L, 18L, 43L, 44L
17	5	49L, 71L, 58L, 62L, 64L
18	1	48L
20	2	53L, 45L
21	1	40L
22	1	36L

Table 5.2 Table showing the 40 *C. difficile* 027 pulsoavar subtypes isolates used in this study

Pulsovar type	No. of isolates	Isolates Ids
I	16	2L, 5L, 14L, 17, 21L, 32L, 38L, 52L, 54L, 61L, 63L 67L, 71L, 75L, 77L, 81L
III	1	48L
IV	23	1L, 3L, 15L, 16L, 18L, 19L, 23L, 30L, 35L, 40L, 42L, 43L, 44L, 45L, 47L, 49L, 53L, 55L, 56L, 58L, 59L, 62L, 64L
V	1	36L

To initiate sporulation from the 41 isolates, an equal amount (500 µl) of 10^4/ml titre of spore sample for each isolate was used. This is to ensure that the same amount of starting spore material was used to initiate sporulation assay for all the isolates and to allow unbiased comparisons of the total spore counts at the end of the experiment. The 10^4/ml concentration was chosen as some of the isolates only produced low concentration of 10^5/ml spores during the purification procedures. Prior to the spore initiation experiment, the spore samples were allowed to germinate in BHI broth supplemented with of L-cysteine and sodium taurocholate for 18 hours. A 200 µl sample was taken from each of the germinating culture and heated at 60 ^{o}C for 10 min to eliminate the vegetative cells. Streaking of the heated sample on BHI agar plates supplemented with L-cysteine and sodium taurocholate and incubation for 18-20 h showed no colony growth. This showed that there was no carryover of spore material from the previous culturing and that isolates were exposed to sporulation all at the same time.

5.3.2 Determination of Spore initiation time

The development of endospores within the 41 *C. difficile* 027 isolates in BHI broth was monitored using light microscopic examination of samples taken at 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h after sporulation. Spore initiation for each isolate was determined by scoring for the presence or absence of endospores at the various time points. Results showed that there was a considerable variation in the time at which the different *C. difficile* 027 isolates initiated endospores production (Table 5.3). It was observed that none of the isolates produced spores at the 0 time point but sporulation was initiated within the first 96 hours of incubation in all the isolates examined. After culturing for 24 h, it was observed that only one isolate, 16L produced endospores. Twenty-one other isolates started producing endospores after 48 h of

incubation. Theseates were 48L, 15L, 18L, 14L, 21L, 81L, 17L, 5L, 75L, 47L, 23L, 36L, 3L, 54L, 58L, 19L, 67L, 77L, 71L, 64L, and 40L (Table 5.3). Seventeen other isolates (1L, 2L, 56L, 42L, 59L, 62L, 44L, 55L, 43L, 45L, 35L, 38L, 52L, 49L, 32L, 61L and 53L) were observed to form endospores after 72 h of incubation (Table 5.3). Spores were observed in two isolates, 63L and 30L after 96 h incubation (Table 5.3).

At the 48 h, it was observed that there was a significant difference in the mean total spore counts produced by isolates that sporulated at this time point (P<0.05, n=21). There were only two isolates that produced spores after 96 h incubation, these were combined with the 17 isolates that produced spores after 72 h incubation and analysed. One-way ANOVA showed that there was also a significant difference in the total amount of spores produced by isolates that sporulated at these time points (P<0.05, n=19) (Appendix 10).

Table 5.3 Spore initiation time and total count of different *C. difficile* 027 strains in relation to their phage carriage

Strain	Time (hours)								Log mean CFU/ml	Morphology of phages induced
	0	24	48	72	96	120	144	168		
16L	-	+	+	+	+	+	+	+	12.20	Myovirus/PT-LPs
48L	-	-	+	+	+	+	+	+	8.41	Siphovirus
15L	-	-	+	+	+	+	+	+	8.45	Myovirus/PT-LPs
18L	-	-	+	+	+	+	+	+	8.23	PT-LPs
14L	-	-	+	+	+	+	+	+	7.60	Myovirus/PT-LPs
21L	-	-	+	+	+	+	+	+	8.46	Myovirus/PT-LPs
81L	-	-	+	+	+	+	+	+	8.58	Myovirus/PT-LPs
17L	-	-	+	+	+	+	+	+	8.37	PT-LPs
5L	-	-	+	+	+	+	+	+	6.88	Myovirus/PT-LPs
75L	-	-	+	+	+	+	+	+	5.59	Myovirus/PT-LPs
47L	-	-	+	+	+	+	+	+	7.62	Myovirus/PT-LPs
23L	-	-	+	+	+	+	+	+	7.65	Myovirus/PT-LPs
36L	-	-	+	+	+	+	+	+	4.77	No phage
3L	-	-	+	+	+	+	+	+	8.70	PT-LPs
54L	-	-	+	+	+	+	+	+	7.68	Myovirus/PT-LPs
58L	-	-	+	+	+	+	+	+	9.21	PT-LPs
19L	-	-	+	+	+	+	+	+	8.31	PT-LPs
67L	-	-	+	+	+	+	+	+	7.48	Myovirus/PT-LPs
77L	-	-	+	+	+	+	+	+	5.61	Myovirus/PT-LPs
71L	-	-	+	+	+	+	+	+	6.53	PT-LPs
64L	-	-	+	+	+	+	+	+	5.57	PT-LPs

40L	-	-	+	+	+	+	+	+	6.60	PT-LPs
1L	-	-	-	+	+	+	+	+	8.15	PT-LPs
2L	-	-	-	+	+	+	+	+	6.56	Myovirus/PT-LPs
56L	-	-	-	+	+	+	+	+	7.44	PT-LPs
42L	-	-	-	+	+	+	+	+	6.49	PT-LPs
59L	-	-	-	+	+	+	+	+	5.76	Myovirus/PT-LPs
62L	-	-	-	+	+	+	+	+	7.77	PT-LPs
44L	-	-	-	+	+	+	+	+	4.49	PT-LPs
55L	-	-	-	+	+	+	+	+	5.68	PT-LPs
43L	-	-	-	+	+	+	+	+	5.79	PT-LPs
45L	-	-	-	+	+	+	+	+	4.46	PT-LPs
35L	-	-	-	+	+	+	+	+	4.81	PT-LPs
38L	-	-	-	+	+	+	+	+	4.72	Myovirus/PT-LPs
52L	-	-	-	+	+	+	+	+	4.51	Myovirus/PT-LPs
49L	-	-	-	+	+	+	+	+	5.65	PT-LPs
32L	-	-	-	+	+	+	+	+	5.69	Myovirus/PT-LPs
61L	-	-	-	+	+	+	+	+	5.68	Myovirus/PT-LPs
53L	-	-	-	+	+	+	+	+	4.71	PT-LPs
63L	-	-	-	-	+	+	+	+	4.66	Myovirus/PT-LPs
30L	-	-	-	-	+	+	+	+	4.54	PT-LPs

PT-LPs: Phage tail-like particles

Spore initiation for each isolate was determined by scoring for the presence (+) or absence (-) of endospores at the various time points. All isolates examined sporulated within the first 96 h: one isolate sporulated within 24 h of incubation, 21 sporulated after 48 h, 17 isolates sporulated after 72 h and 2 isolates after 96 h of incubation. All experiments were performed in duplicate on two separate occasions.

5.3.3 Comparative spore counts of *C. difficile* 027 strains

At the end of the 168 h incubation, the total spores produced by each of the isolates were purified using HistoDenz density gradient centrifugation and afterwards heat-treated at 60 $^{\circ}$C 20 min. The heat resistant spores were enumerated by determining their average viable counts from three replicate plate counts. It was observed that there was a significant variation in the total amount of spores produced by the isolates examined. Strain 16L was the highest spore former with an average concentration of 10^{12} CFU/ml spores produced (Figure 5.2). Interestingly, this isolate was also the first to initiate spore production (Table 5.3). Ten other isolates (48L, 15L, 18L, 21L, 81L, 17L, 3L, 58L, 19L and 1L) produced spores ranging from 10^8-10^9 CFU/ml spores. These ten isolates sporulated after 48 h except 1L which started producing spores after 72 h incubation. Twelve isolates (14L, 5L, 47L, 23L, 54L, 67L, 71L, 40L, 2L, 56L, 42L and 62L) produced a spore titre of 10^6-10^7 CFU/ml. The isolates initiated spore formation after 48 h incubation except 2L, 56L, 42L and 62L which initiated spore production after 72 h. The remaining majority of the isolates (18 isolates) produced spore titres ranging from 10^4 -10^5 CFU/ml (Figure 5.2); ten and eight isolates produced spores of 10^5 CFU/ml and 10^4 CFU/ml respectively. Among the ten isolates with the 10^5 CFU/ml spore concentration, three (75L, 77L and 64L) initiated spore production after 48 hour incubation and seven (59L, 44L, 55L, 43L, 49L, 32L and 61L) initiated spore production after 72 h incubation. The eight isolates (36L, 45L, 35L, 38L, 52L, 53L, 63L and 30L) that produced the lowest amount of spores (10^4/ml) initiated spore production within 72 and 96 h incubation except 36L which produced spores after 48 h incubation. In summary, this result revealed that early spore initiation in the isolates did not necessarily result to high spore count at the end of the 168 h in all the isolates.

One-way ANOVA revealed that there was a significant difference in the mean concentration of the spores produced by all the isolates used in this experiment ($P<0.05$, n=41). This shows that there is a significant variation in the amount of spores produced by isolates within the 027 ribotype. Accordingly, no significant differences were observed in the three replicates of the spores produced by each of the isolates tested ($P>0.05$).

The total spore counts were also analysed based on their MLVA and Pulsovar subtypes. One-way ANOVA showed there is a significant difference in the mean spore counts among the 16 isolates that are found in Pulsovar I ($P<0.05$, n=16). Similar result was obtained with Pulsovar IV ($P<0.05$, n=23) (Appendix 10). It was not statistically possible to analyse the samples based on their MLVA types as many of the MLVA types (6/13) have just one representative isolates. In addition, no correlation was observed between spore initiation times and the subtypes. Similar observation was made with the mean total spore count.
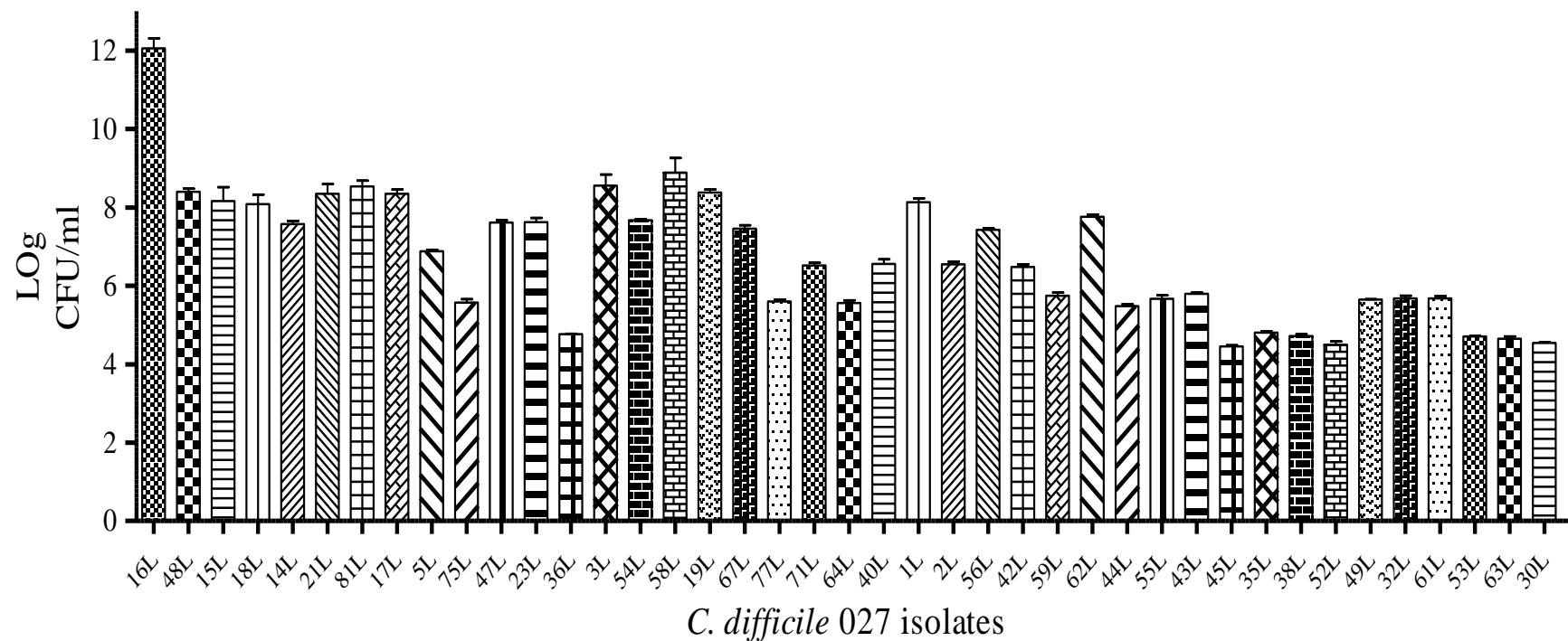
Figure 5.2 Total spore counts of *C. difficile* 027 isolates after 168 h incubation

The total spores produced by the 41 *C. difficile* isolates at the end of 168 h incubation were purified with HistoDenz density gradient and heat treated at 60°C for 20 min. The heat resistant spores were enumerated by determining their viable counts from triplicate plate counts on BHI supplemented agar. The experiment was carried out twice. The total spore counts were analysed using One-way ANOVA (Analysis of Variance) in GraphPad Prism 5 (Appendix 10). Statistical significance was indicated by a P value of 0.05. Error bars represent standard errors.

5.3.4 Spore counts in relation to phage carriage

The spore counts of the isolates examined were compared to their phage carriage. Among the 41 isolates, one isolate harbour siphoviruses, 18 harbour myoviruses/phage tail-like particles, 21 harbour only phage tail-like particles and one isolate contain no phage after induction with mitomycin C and norfloxacin (Table 5.3). It was observed that the one isolate that produced the highest spore count and started sporulating within the first 24 h of incubation harbour a novel myovirus E. Among the 21 isolates that began forming spore by the 48th hour, about 52 % (11/21) harbour myoviruses and PT-LPs, 38 % (8/21) contained PT-LPs only and 5 % (1/21) harboured siphoviruses (Table 5.3). In interestingly, 36L which contain no phage was the only strain that produced the lowest spore count at this time point. After 72 h incubation, 65 % of the isolates that produced spore at this time point harbour PT-LPs in their genomes. Finally, among the two isolates that formed spores after 96 h incubation, one isolate (63L) contain a myovirus and PT-LPs and the other (30L) contain only PT-LPs after prophage induction.

5.3.5 Light microscopy examination of endospores

Light microscopy examination of the Gram-stained slides showed that the Gram-positive *C. difficile* rod-shaped vegetative cells vary in size and ranged between ~4 to 5 µm long and 1-1.2 µm wide. By the end of 48 h of incubation, the vegetative cells showed Gram-variability due to osmotic and dehydration stress on the cell wall. The endospores produced were observed to be terminal or sub-terminal, occupy about 1/3 of the rods and stained lighter than the rest of the vegetative part of the cells (Figure 5.3). It was also observed that about 40 % of the spores were still attached to their mother cells by the 120 h of incubation. By the 144 h and 168 h of incubation however, about 90 % of the spores have matured and were observed to have detached from their mother

cells in all the isolates examined. Phase contrast microscopy of spores from the broth cultures showed that the spores are dark brown or black in colour and assumed a uniformly elliptical shape.
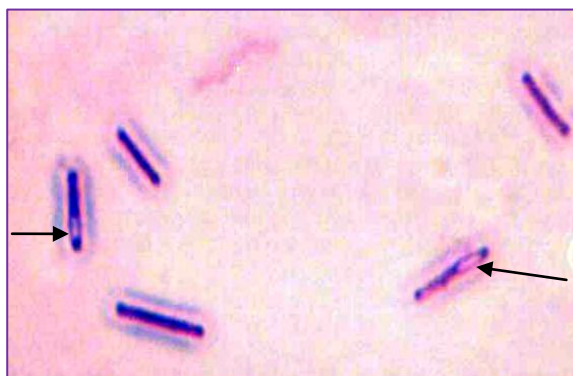
Figure 5.3 *C. difficile* vegetative cells and endospores as observed under a light microscope.

V = A Gram-positive rod-shaped vegetative cell, E = A spore mother cell showing terminal endospore. Arrows point to the endospores.

5.3.6 Sporicidal activities of three disinfectants on *C. difficile* spores

Fifteen isolates were selected for scanning electron microscopy (SEM) analysis of their spore external morphology. The isolates were selected to cover the different MLVA types examined. Before the spores were analysed using SEM, they were inactivated with 2 % glutaraldahyde, 37-40 % formaldehyde or presept 1000 ppm. Results showed that there was appoximately 1-2 log reduction in viable spore count at 0 min with glutaraldehyde and presept as verified by the colony counts on BHI agar medium supplemented with L-cysteine and sodiun taurocolate. After 30 min of incubation before plating, the spores were completely killed with glutaraldehyde and presept treatments (Table 5.4). With formalin however, there was complete inactivation of spores from 0 min as confirmed by the colony count (Table 5.4).

Table 5.4 Table showing killing effect of glutaraldehyde, formalin and presept on *C. difficile* spores

| Isolate | Control | Number of surviving spores (CFU/ml) at various time points (min) after disinfectant treatment | | | | | | | | | | | |
| | | 2 % Glutaraldehyde | | | | 37-40 % Formaldehyde | | | | Presept 1000 ppm | | | |
		0 min	30 min	60 min	120 min	0 min	30 min	60 min	120 min	0 min	30 min	60 min	120 min
16L	8.0×10^4	1.3×10^2	0	0	0	0	0	0	0	3.6×10^3	0	0	0
15L	2.4×10^4	1.9×10^2	0	0	0	0	0	0	0	2.1×10^2	0	0	0
2L	4.2×10^4	3.1×10^3	0	0	0	0	0	0	0	2.6×10^2	0	0	0
36L	4.1×10^4	3.2×10^3	0	0	0	0	0	0	0	2.9×10^2	0	0	0
17L	1.8×10^4	8.5×10^2	0	0	0	0	0	0	0	4.4×10^2	0	0	0
3L	4.02×10^4	2.9×10^2	0	0	0	0	0	0	0	2.3×10^2	0	0	0
48L	1.1×10^4	9.0×10^2	0	0	0	0	0	0	0	1.4×10^2	0	0	0
40L	6.23×10^4	4.71×10^2	0	0	0	0	0	0	0	3.38×10^2	0	0	0
67L	2.12×10^4	1.75×10^2	0	0	0	0	0	0	0	4.5×10^3	0	0	0
81L	1.92×10^4	1.61×10^2	0	0	0	0	0	0	0	1.2×10^3	0	0	0
54L	3.8×10^4	3.2×10^2	0	0	0	0	0	0	0	1.4×10^2	0	0	0
43L	2.28×10^4	1.97×10^2	0	0	0	0	0	0	0	1.29×10^2	0	0	0
91L	9.9×10^4	7.1×10^3	0	0	0	0	0	0	0	3.5×10^2	0	0	0

Spore suspensions were inactivated with 2 % glutaraldahyde, 37-40 % formaldehyde or presept 1000 ppm before being subjected to SEM analysis. There was 1-2 log reduction in viable spore count at 0 min with glutaraldahyde and presept treatments. There was complete inactivation of spores at 0 min incubation with formalin and after 30 min incubation with glutaraldahyde and presept. All experiments were performed in duplicate on two separate occasions.

5.3.7 SEM analysis of *C. difficile* spores

SEM analysis of glutaraldehyde-inactivated *C. difficile* spores revealed that they were ellipsoidal in shape and measured ~2.5 x 1 µm in size. Generally, the spores are characterised by a very thick exosporial coat uniformly covered by protrusions indicated using black and blue arrows (Figure 5.4A and B). The spores have one of two basic morphological profiles. The spores with the first morphology (Morphology A) have rounded rectangle ends (A1 and A2). Out of the 15 isolates spore preparations that were examined, 12 had this morphology. However, within the Morphology A, there were slight variations in the thickness of the exosporial protrusions. There are spores with thick protrusions that appeared to form contours or lobes indicated by black arrows on the surface of the spores (Figure 5.4A), and there are those that have very thin protrusions that formed projections and are needle-like in appearance as shown using blue arrows in Figure 5.4B. Of the 12 isolates that bore spores with the morphology A, 4 had the subtype morphology A1 and 8 bore the subtype morphology A2 (Figure 5.4). The second spore morphology (Morphology B), is also characterised by a thick exosporial spores membranes with protrusions resembling that of Morphology A1. However, one of the ends is markedly smaller than the other and the larger end is rounded and appeared to be swollen. The thin and thick ends are separated by a constriction indicated using a green arrow (Figure 5.4B). Therefore, the whole structure of morphology B results to a bottleneck shape. Only one isolates 48L, showed this morphology.

A few spores were observed to have started germinating. During early stages of germination, the lobes and protrusions disappeared due to the absorption of moisture and spore membrane become smoother in appearance (Figure 5.4C). In addition, a small delicate protrusion, (indicated using a red arrow) growing outwards from one end

of the germinating spore was observed (Figure 5.4C). There was no instance in which one or more of the protrusions were observed to grow from one or both ends. These protrusions will later grow into a germination tube from which the vegetative cells arise.
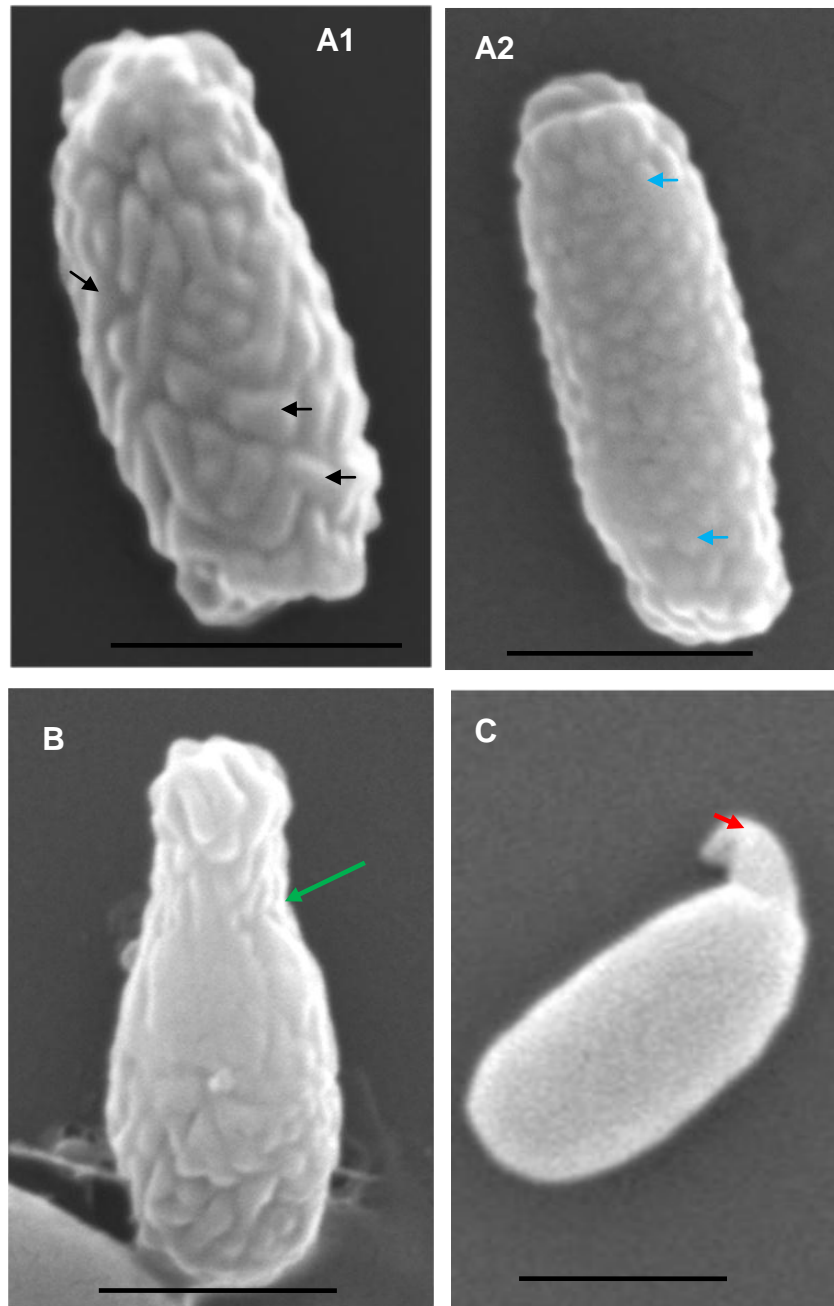
Figure 5.4 SEM images of *C. difficile* spores. Bars ~1 µm

Above are the SEM images of the *C. difficile* spores examined in this study. The spores are ellipsoidal and measured ~2.5 x 1 µm. They are characterised by a thick exosporial coat and uniformly covered by protrusions which may be thick or needle-like represented by black and blue arrows respectively (Figure 5A1 and A2). The spores have two basic morphological profiles. The spores with the first morphology have rounded rectangular ends observed in ~80% of the isolates (Figure 5A1 and 3A2). In the second morphology (observed in ~20%), one of the ends is markedly smaller than the other and the larger end appeared to be swollen. The thick and thin ends are separated by a green arrow (Figure 5B). During early stages of germination, the lobes and protrusions disappeared due to the absorption of moisture and a small delicate protrusion (indicated using a red arrow) growing outwards from one end was observed (Fig. 3C).

5.4   Discussion

The *C. difficile* 027 ribotype can be subdivided into subclades which have been reported to vary in their severity of CDI (Fawley *et al.*, 2008). There is a great need to understand the role of contributory factors that may be responsible for the diversity and pathogenicity within the ribotype which leads to their success and re-emergence in the hospital and community settings. Sections 3 and 4 of this thesis have shown that diverse morphologies of phages are associated with this ribotype and correlates to the different subclades (Nale *et al.*, 2012). Work carried out in my thesis has provided evidence to suggest that the phages do contribute to the diversity and subsequently to the pathogenicity of these strains. In the sporulation study, I expanded these efforts to consider another factor, sporulation, which may also play a significant role in the pathogenicity of this ribotype. One phage isolated and characterised in our laboratory was found to contain the *agrDBCA* complex and histidine kinases that can influence sporulation and quorum sensing (Kate Hargreaves Pers Comm). The agr quorum sensing has been reported to regulate sporulation in *C. acetobutylicum* (Steiner *et al.*, 2012). Therefore, this study compared sporulation rates among *C. difficile* 027 subclade isolates and correlated this to the severity of disease associated with the subclades and their phage carriage. It was found that many of the isolates produced a significant high amount of spores. However, the spore initiation time and total spore counts varied among the isolates but did not correlate to the subtypes or the phage carriage within the strains.

Molecular insight into the genome of *C. perfringens* temperate bacteriophage phi3626 (a siphoviurs) showed the presence of genes encoding a putative sigma factor related to sporulation-dependent sigma factors and a putative sporulation-dependent transcription regulator (Zimmer *et al.*, 2002). Therefore, it was suggested that the phage

might interaction with the onset of sporulation in *C. perfringens.* In another study, phage carriage from *C. difficile* isolates was correlated to disease severity among patients attending University Hospitals of Leicester (Tromans *et al.*, 2010). The report showed that increased disease severity was associated with strains containing siphoviruses of capsid size ~70 nm. However, no further evidence to show in what way the phages could affect the pathogenicity of the *C. difficile* (Tromans *et al.*, 2010). Due to these two reasons, sporulation in the isolates examined was correlated to phage carriage to determine whether there is any link between the observed sporulation and phage morphologies they harbour. It was observed that there was no clear correlation between spore initiation time and total spore yield with phage carriage although majority of strains with specific phage carriage produced spores at certain time points. For example, it was observed that 11/21 isolates (52 %) that produced spores at the 48th hour harbored myoviruses and PT-LPs and 11/17 (65 %) isolates that produced spores at the 72 h harbored PT-LPs only. In addition, the only isolate 48L that harbored siphoviruses was observed to start forming spores at the 48th hour of incubation. Interestingly, isolate 36L which contained no prophage was found to yield spores of equal concentration as those that harbored other morphologies of phages. This results show that there is no association between phage carriage and sporulation in *C. difficile* 027, thus suggesting that rate of spore production in *C. difficile* does not depend on the phage contents but may depend on other factors in the individual isolates (Burns *et al.*, 2010a).

Spores are the most likely form of *C. difficile* in the hospital and community environment (Roberts *et al.*, 2008). These resistant structures do not only provide means of protection but act as vehicles of transmission to susceptible patients through contact with the environment, healthcare or visitors (Riggs *et al.*, 2007, Alfa *et al.*,

2008). Therefore, *C. difficile* strains that are capable of sporulating easily or produce a high amount of spores are of great of concern. Previous studies have compared sporulation rates among different *C. difficile* ribotypes and showed that there is no significant difference in the amount of spores produced by the 027s from the non-027s strain (Burns *et al.*, 2010a, Burns *et al.*, 2011). However, when sporulation rates of three ribotypes of *C. difficile* was compared by another group of researchers, the ribotype 027 was found to be significantly higher (Wilcox and Fawley, 2000). Furthermore, several reports have also showed that the *C. difficile* 027 strains have great sporulation efficiency compared to other ribotypes (Akerlund *et al.*, 2008, Rupnik *et al.*, 2009, Merrigan *et al.*, 2010, Dingle *et al.*, 2011, Vohra and Poxton, 2011). The controversy in these reports lies in the sample sizes which was suggested to be small in the later reports (Burns *et al.*, 2011). Therefore this study addressed this issue by analysing the sporulation characteristic of 41 different *C. difficile* 027 isolates. This forms the largest population size ever examined in this area of research. Thus, the results obtained here will greatly support previous evidence of high sporulation rates in *C. difficile* 027 strains.

In this study, it was shown that there is considerable variation in the rate of spore initiation and formation among the 027 strains examined. It was observed that many of the isolates initiated spore production at an early stage of incubation (within the first 24 h and 48 h) and a concomitant high spore yield. The early spore formation observed in this study is in agreement with previous report where hypervirulent strains were found to produce spores earlier than the non-hypervirulent ones (Merrigan *et al.*, 2010). As an advantage, early and enhanced sporulation provides protection in these strains and could increase their chances of escape from the harsh environmental conditions (Setlow, 2007). Thus, enabling them to cope with nutrient deficiencies and toxin

accumalation, persist better and increase their likelihood of dissemination form the environment (Akerlund *et al.*, 2008). In addition, the 18 and 1 bp deletions in the *tcd*C gene leading to increased toxin production previously reported in these strains coupled with increase sporulation may have combined to the observed high disease severity associated with some of the strains (Warny *et al.*, 2005, Akerlund *et al.*, 2006, Merrigan *et al.*, 2010).

Beside mutations in the *tcd*C, sporulation in *C. difficile* has been shown to be accompanied by high toxin yield. Studies have shown that spore detecting determinants in *C. difficile* are directly related to toxin production (Heap *et al.*, 2007, Underwood *et al.*, 2009). The inactivation of the master spore determinant, Spo0A and a putative spore regulator, histidine kinase also found in phage resulted in the production of an asporogenous mutant and a 3.5 fold spore reduction respectively. In addition, inactivation of both regulators showed marked decrease in toxin production (Castilla-Llorente *et al.*, 2006, Castilla-Llorente *et al.*, 2009, Underwood *et al.*, 2009). Although Akerlund *et al.* (2006) reported an inverse correlation of *C. difficile* toxin to sporulation; they attributed the higher toxin levels detected in some of the isolates to other undetected *C. difficile* that may be present in the faeces and the low spore germination to the CCEY medium. The CCEY medium was reported to have a lower efficiency to spore recovery compared to the sodium taurocholate supplemented medium (Wilson *et al.*, 1982, Buggy *et al.*, 1983, Buggy *et al.*, 1985, Bliss *et al.*, 1997). These evidences suggest that the high sporulation in the *C. difficile* 027 isolates observed in this study may have a simultaneous effect on their toxin levels and thus may account for the high disease severities observed.

Although the rate of spore initiation was determined by phase contrast microscopy and light microscopy, the enumeration of the total spore counts was done

by analysing the heat resistant spores and CFU/ml count as previously suggested (Burns *et al.*, 2011, Burns and Minton, 2011). The combination of Gram-staining and light microscopy to monitor sporulation as used in this study was found to be particularly useful in distinguishing the vegetative cells from the endospore as well detecting possible contamination during the experiment. A recent study used heat resistant CFU/ml spore counts to monitor sporulation in *C. difficile* (Burns *et al.*, 2011). However, to determine heat resistant spores at an early germination time may mean that some of the young spore may be lost during the heating process and this might not be accounted for in the CFU/ml count. In addition incomplete heat treatment and the sensitivity of the endospores to different growth medium may lead to erroneous result (Burns and Minton, 2011).

It was observed that there was a significant variation in the amount of spores produced by the 027 ribotype strains. This result concurs with other reports where variable spore counts were reported within the 027 strains (Warny *et al.*, 2005, Akerlund *et al.*, 2008, Burns *et al.*, 2011). Although some of the strains produced low spore count (10^4 CFU/ml), about 24 % of the isolates examined produced 10^{8-12} CFU/ml spore. This is the highest spore concentration ever reported and is higher than all other ribotypes spore counts previously reported (Wilcox and Fawley, 2000, Akerlund *et al.*, 2008, Burns *et al.*, 2010a, Burns *et al.*, 2011, Vohra and Poxton, 2011). Therefore, this chapter strongly suggests that the ribotype 027 is greatly associated with early spore initiation and high spore yield compared to other ribotypes.

The purification of the *C. difficile* spores using HistoDenz density gradient centrifugation may account for purity as well as the high spore concentration observed (Sorg and Dineen, 2009, Hudson *et al.*, 2011). HistoDenz (5-(N-2,3-Dihydroxypropylacetamido)-2,4,6-triiodo-N,N'-bis(2,3-dihydroxypropyl)

isophthalamide, molecular formular $C_{19}H_{26}I_3N_3O_9$), is a non-ionic X-ray contrast compound developed specifically as a gradient medium for separating macro and micro molecules by rate-zonal or isopynic centrifugation (Landers, 2008, Sakanaka *et al.*, 2008). Sucrose density gradient work in the same way and has previously been employed in the purification of *C. difficile* spores (Lawley *et al.*, 2009). Other previous reports on *C. difficile* spore isolations did not involved any means to purify their spores. Therefore, only growing cultures at certain optical density values were used to initiate sporulation for 2-5 days and the resultant heat resistant spore samples were serially diluted and plated. The CFU/ml count was done from the plates (Wilcox and Fawley, 2000, Akerlund *et al.*, 2006, Akerlund *et al.*, 2008, Burns *et al.*, 2010a, Burns *et al.*, 2011). Merrigan *et al.* (2010) washed the vegetative cell with spores several times with phosphate buffer solution (PBS) after which the cells were heated, serially diluted and plated. It is possible that a lot of the spores might have been lost during the process of PBS washing thus accounting for the low spore count. Therefore, purification of spores using HistoDenz density centrifugation separates the spores from vegetative and dead cells resulting to the production of pure spore samples as previously reported (Sorg and Dineen, 2009).

To ensure that the same amount of starting bacterial material was used to initiate sporulation, an approximately equal amount of spores was used. An equal amount of starting material is necessary for an unbiased comparison of sporulation among these isolates (Vohra and Poxton, 2011). To initiate sporulation from an overnight culture may introduce spores from the previous culturing and this may suggest that sporulation might have been initiated prior to the start of the experiment. Previous report have added bile salts to growing *C. difficile* culture to enhance germination and ensure that minimum spores were sustained before initiating sporulation (Wilson *et al.*, 1982, Sorg

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and Sonenshein, 2008, Heeg *et al.*, 2012). However, this did not ensure complete germination as heat resistant spores were still detected after culturing on brain heart infusion supplemented (BHIS) medium with bile salt taurocholate (Burns *et al.*, 2011). As germination of spore vary from strain to strain, it is important to ensure that the there is complete germination by adjusting the concentration of germinant used or increase the incubation time. In addition, it is important that the effect of the germinant be removed by washing the bacterial/spore sample and re-suspending same in a fresh medium without the germinant as demonstrated in this study.

The sporulation time of the isolates used in this study was allowed for up to 168 h (7 days). Although the exact period of complete germination in *C. difficile* is not known, it was suggested that the process is generally completed after 120 h of incubation (Burns *et al.*, 2010a, Burns *et al.*, 2011). However, this duration was still found to be insufficient as most of the isolates could not reached their maximal spore count at this time point (Burns *et al.*, 2010a). Therefore, this study has allowed an additional 48 h to allow maximum spore formation and this might also contribute to the high spore titre observed in this study as previously reported (Lawley *et al.*, 2009).

The sporicidal effect of three disinfectants on *C. difficile* spores was compared. We found glutaraldehyde, precept and formalin to effectively kill *C. difficile* spores although formalin had greater sensitivity with the killing recorded at the 0 min of incubation. Although glutaraldehyde has been used for sterilization of hospital equipment in the past, it has been to be unsafe to use (Babb *et al.*, 1980, Cowan *et al.*, 1993, Stevens, 1994). Notwithstanding, under strict safety precautions, glutaraldehyde has been used as fixative for TEM and SEM specimens (Dyas and Das, 1985, Lawley *et al.*, 2009). Furthermore, due to its inability to damage plastics, metals and rubber, glutaraldehyde could be employed in industrial settings where certain equipment cannot

be autoclaved (Kohlpaintner *et al.*, 2000). Although it was observed that presept and formalin were effective in eradicating *C. difficile* spores, however their effect on preserving the spore morphology has not been verified in this study. Further study is therefore required to ascertain their suitability as fixative agents of *C. difficile* spores.

5.5   Conclusion and Future work

This study shows that the *C. difficile* ribotype 027 strains produce 10^{4-12} CFU/ml spores. Although the spore initiation times and spore concentrations vary significantly among the isolates examined, however they did not correlate to their subtypes or phage contents. The early and enhanced sporulation among some of the isolates enables them to withstand nutrient deficiencies and toxin accumulation. In addition, the spores enable them to persist better and increase their likelihood of dissemination from the environment. Furthermore, the high sporulation efficiency coupled with high toxin production significantly contributes to the hypervirulence nature of strains within this ribotype and their success in the environment. Future work will focus on genome sequencing to explore the role of genes in the pathogenesis of CDI.

6 Summary

The hypervirulent *Clostridium difficile* PCR ribotype 027 is one of the most clinically relevant ribotypes in the UK and in many parts of the world. Infection due to this ribotype has contributed significantly to the economic and clinical burden of CDI in the UK. The pathogenicity of *C. difficile* 027 is well characterised; it encodes a binary toxin gene and has deletions in the *tcd*C gene which causes high toxin production. Furthermore, *C. difficile* 027 strains are fluoroquinolone resistant and sporulate readily, both of which contribute to the success of this pathogen. Interestingly, this ribotype can be divided into at least 23 MLVA based subclades and 5 Pulsovar types. These subtypes have been reported to vary in their severity of infection. It is therefore important to establish other factors that may be contributing to the diversity and success of this hypervirulent ribotype and the epidemic potential of new strains. One such factor is bacteriophage carriage. To determine whether temperate bacteriophages are contributing to the *C. difficile* diversity and phenotype, the range of bacteriophages present in clinically relevant strains such as ribotype 027 must first be established. In this study, temperate bacteriophages associated with the *C. difficile* 027 subtype isolates were induced and characterised, and their contribution to diversity was determined.

Although genomic analysis of *C. difficile* 027 strains has shown that they encode mobile genomic elements including prophages, only one inducible phage was reported to be associated with this ribotype. In addition, it was also reported that a particular ribotype harbors only a particular morphology of phage. Thus, it was previously suggested that prophage content is an indication of ribotype. However, the induction of 91 ribotype 027 isolates and TEM analysis of the induced lysates in this study showed that they encode for phages with diverse morphologies. This study therefore, changes the previously published paradigm of one phage one ribotype.

The morphologies of the tailed bacteriophages induced belong to Family *Caudovirales* and included two Orders: the *Myoviridae* and the *Siphoviridae* and phage tail-like particles (PT-LPs). Myoviruses were induced from 62 isolates with basic subtypes morphology identified. The first was the intact myoviruses (Morphology A) which were induced from two isolates only. This morphology with a capsid of ~70 nm was the only morphology previously reported to be induced from a ribotype 027 strain. However, the tail length of the phage induced in this study (of ~200 nm by 20 nm) is longer than the previously described phage (~160 nm/20 nm). The second subtype of myoviruses induced was the 'defective' myoviruses (Morphologies B, C and D). They were characterised by a hexagonal capsid (~70 nm) but the tails were constricted at different positions on the tail tube. The defective myoviruses were induced from 59 isolates as the only viral particles, from two isolates which also yielded the typical myoviruses and from one other isolate yielding siphoviruses. The defective myoviruses were previously reported to be associated with mitomycin C induced lysates and in the same lysate that contained typical myoviruses. However, the defective myoviruses were found in majority of both mitomycin C and norfloxacin induced lysates examined as the only phage morphology and in other isolates that harbour other phage morphologies. Therefore, this finding suggests that dual phage carriage exist in these strains. The third myoviruses morphology is the novel myovirus E induced from one isolate. This morphology bears close resemblance to the typical myoviruses except that the capsid (of ~40 nm) was quite smaller in size. Although this phage morphology is similar to 'killer particles' found in *Bacillus* species, *Acetobacter* species and *Clostridium botulinum*, this report is the first to show the induction of this phage with this morphology from *C. difficile*. This kind of phages are thought to package host DNA instead of bacteriophage DNA and are able to kill sensitive hosts but not replicate

within them. My work agrees with this report as fresh induced samples of myovirus E could clear lawns CD630 during plaque assay but the plaques could not further propagate, and TEM analysis identified only broken tails from the plaques. The ability of this phage to clear lawns of CD630 is particularly interesting, however, more investigations are required to further characterise it and its potentials for phage therapeutic purpose. Due to the very narrow host range of the temperate bacteriophages induced from the clinical isolates in this study, they may be unsuitable for *C. difficile* phage therapy. Therefore, lytic phages from the environment or temperate phages with lytic ability from environmental *C. difficile* strains may be investigated for this purpose.

Siphoviruses were induced from three isolates and have a capsid of ~70 nm in diameter and a flexible tail of ~230-350 nm. Although identical siphoviruses with similar dimensions have previously been reported on other ribotypes of *C. difficile*, this report is the first to show the association of this phage morphology with ribotype 027.

Dual phage carriage was identified in four isolates. Two isolates harboured typical myoviruses and defective myoviruses, one yielded defective myoviruses and siphovirures and one other isolate contained two morphologies of siphoviruses that deferred in their tail lengths. This report is again the first to show two inducible phages in ribotype 027 although similar finding has been reported in ribotype 012 (strain CD630).

To determine if the phages are contributing to the diversity of ribotype 027, the diverse phage morphologies were correlated to the MLVA and Pulsovar types of the host isolates. A correlation between the subtype morphology was observed. About 74 % (17/23) and 40 % (2/5) of the MLVA and Pulsovar types contained a specific phage respectively. Therefore, statistically relevant correlation was observed with the MLVA

types. It was therefore strongly suggested that the phages are indeed contributing to diversity.

The phages also differed in their whole genome size which ranged between 15-50 kb and fall within the range of genome sizes of *C. difficile* phages.

Two different antibiotics mitomycin C and norfloxacin were used to induce prophages from the *C. difficile* isolates. The antibiotics were found to induce specific prophages from their hosts. Therefore it was suggested that the modes of action of the antibiotics might contribute significantly to the observed differences in the phages induced. Although norfloxacin was previously used to induced prophages from *E. coli*, it was found to be effective in inducing prophages from *C. difficile* also and led to the induction of ~30 % of the isolates that wouldn't have been induced with mitomycin C alone. During induction, the growth responses of the bacterial cultures were monitored by measuring their OD_{550} values before and after induction. At the end of 24 h induction, four different patterns of growth responses were observed. The four responses included a drop in OD_{550} values with both antibiotics, a rise with norfloxacin and drop with mitomycin C, a rise with mitomycin C and a drop with norfloxacin and a situation in which the OD_{550} values of bacterial cultures with both inductions remained relatively constant. In addition, it was observed that a drop in OD_{550} values did not always correlate with phage release. However, after 120 h incubation of 12 cultures from the different patterns, they were found to show a significant drop in OD_{550} values and a concomitant release of phages.

The characterisation of temperate bacteriophages is cumbersome due to the limitation of finding the right inducing agents and susceptible hosts to propagate them as observed in this study. In addition, the TEM analysis of the phage morphologies is time consuming. Therefore, a new molecular approach using PCR assay was designed

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to identify and characterise *C. difficile* phages. Since the majority of the induced phages were myoviruses, molecular markers in form of PCR primers targeting three relatively conserved phage genes (capsid, holin and portal) were designed for this group of phages. Analysis of the gene sequences showed that the 027 ribotype strains have highly conserved genes which differed from other ribotypes and bacteria species. The capsid primers were not able to detect some phages that do not have a coverage major capsid gene and the portal protein primers were too degenerate and did not detect phages with unrecognisable portal genes. The holin which is covered in all *C. difficile* strains was found to be a much more suitable molecular marker as an indicator of phage presence because it has the ability to detect all phages. These markers, whether used singly or in combination will greatly help in detecting phage presence and strain choice before the time consuming prophage induction and TEM analysis. Phage genome sequencing will help refine primer sets to target other *C. difficile* phage morhologies but my report has shown that the holin gene is most suitable marker to study *C. difficile* myoviruses.

C. difficile phages were shown to encode the histidine kinase gene which evokes a global regulatory role including toxin production and sporulation (Kate Hargreaves Pers Comm). Therefore, sporulation rates among 41 of the 91 *C. difficile* 027 isolates were determined and correlated to their subtypes and prophage carriage. Sporulation was initiated from spore-free bacterial cultures for 168 h. It was found that all the ribotype 027 isolates examined started producing spores within the first 96th hour. In addition, they produced a significant amount of spores which varied statistically among the isolates but did not correlate to the subtypes or phage carriage. It was suggested that the observed early spore initiation and high spores produced by these isolates could provide them with protection and an escape mechanism from unfavourable conditions. The high

sporulation efficiencies of the 027 strains with high toxin production may significantly contribute to their hypervirulence nature and success in the environment. Future work will focus on genome sequencing to explore the potential role of phages and genes in the pathogenesis of CDI.

In conclusion, this study has provided substantial evidences of two factors, phages and sporulation as major key players to the diversity, evolution and success of *C. difficile* 027 strains.

7 Appendices

7.1 Appendix 1. Media, Buffers and Solutions

Media

All autoclaving was done at 121 ^{o}C for 15 min.

1. **Brazier's selective medium (CCEY)**

   Brazier's powder medium (CCEY Agar) …………………….. 56.5 g

   Distilled water…………………………………………………..1 L

   Dissolve and autoclave. Allow to cool to about 47 ^{o}C before adding 50 ml of S2085 sterile egg yolk tellurite emulsion, 10 ml each of cyclosterine (250 mg/l) and cefoxitin (8 mg/l). Mix and dispense unto plates. Store at 4°C.

2. **BHI 7 % blood agar**

   Brain heart infusion (BHI) powder…………………………………..37 g

   Agar No 1……………………………………………….................10 g

   Distilled water………………………………………………………..930 ml

   Autoclave. Allow to cool to 47 ^{o}C.

   Add 70 ml of defibrinated horse blood, mix before and dispense unto plates. Store at 4°C.

3. **BHI broth**

   BHI…………………………………………………………………..37 g

   Distilled water ………………………………………………………1 L

   Autoclave. Store at 4°C.

   Meat broth (Prepare in Bijou bottles)

   Cooked meat granules………………………………………….0.75 g

   BHI broth…………………………………………………………10 ml

Autoclave. Store at room temperature.

4. **Ultra pure (UP) water**

Add certain amount of nano pure water in Duran bottle. Autoclave.

5. **5 % Chelex 100 in 5 ml**

UP water …………………………………………………………… 5 ml

Chelex resin …………………………………………………...0.25 g

Use immediately or store at 4^oC.

6. **Fastidious anaerobe broth in 1 L**

Fastidious anaerobe broth powder…………………………..........45.7 g

Distillled water…………………………………………………… 1 L

Autoclave. Store at 4^oC.

7. **Luria Bertani (LB) Broth in 1 L**

Tryptone…………………………………………………………10 g

Yeast Extract …………………………………………………5 g

NaCl …………………………………………………………10 g

 Distilled water …………………………………………………1 L

Autoclave. Store at 4^oC.

8. **LB Agar medium in 1 L**

Tryptone………………………………………………………..10 g

Yeast Extract ………………………………………………..5 g

NaCl …………………………………………………………...10 g

 Agar No 1…………………………………………………...15 g

Distilled water …………………………………………………1 L

Autoclave. Allow o cool to about 50 ^oC and dispensed unto plates. Store at 4^oC.

9. **LB Agar medium supplemented with 50 μg/ml of Ampicillin/Kanamycin in 1 L**

   Prepare LB agar as above. Allow to cool to 50 ^{o}C.

   Add 10 ml filter steriled 50 mg/ml (50 mg/ml w/v in UPH$_2$O)

   Ampicillin/Kanamycin was added before dispensing into plates. Store medium

   at 4 ^{o}C and use within two weeks.

10. **LB Agar medium supplemented with 50 μg/ml of Ampicillin/Kanamycin**

    Prepare LB broth as above. Allow to cool. When needed add 1 ml filter steriled

    Ampicillin/Kanamycin (50 mg/ml w/v in UPH$_2$O) to 100 ml and use

    immediately.

11. **SOC medium (0.5 % Yeast Extract, 2 % Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose).**

    Tryptone ……………………………………………………2 g

    Yeast extract ……………………………………………….0.5 g

    NaCl ………………………………………………………0.05 g

    1 M KCl…………………………………………………... 0.25 ml

    Distilled water…………………………………………….to make up to

    100 ml

    Autoclave. Store at room temperature.

Buffers

1. **SM buffer (10 mM NaCl, 8 mM MgSO$_4$.7H$_2$O and 50 mM Tris-Cl) in 1L**

   NaCl ……………………………………………………… 5.8 g

   MgSO$_4$.7H$_2$O………………………………………………….2 g

   1 M Tris-Cl………………………………………………….50 ml

Distilled water…………………………………………………………to make up 1000 ml

Autoclave. Store at room temperature.

2. **Double salt solution for agar sloppy in 100 ml (1 M of MgCl, MgSO$_4$, CaCl$_2$ and NaCl)**

MgCl$_2$…………………………………………………..…... 20.33 g

CaCl$_2$…………………………………………………..14.70 g

MgSO$_4$…………………………………………………….24.65 g

NaCl………………………………………………………5.84 g

Distilled water ……………………………………………to make up 100 ml

Autoclave. Store at room temperature.

3. **20 x SSC buffer in 100 ml**

NaCl…………………………………………………….17.53 g

Sodium citrate …………………………………………........8.82 g

Distilled water ……………………………………………..50 ml

Adjust pH to 7.0

Distilled water ……………………………………………….to make up 100 ml

Autoclave. Store at room temperature.

4. **0.1 x SSC buffer in 100 ml**

20 x SSC…………………………………………………………500 µl

UP H$_2$O…..……………………………………………99.5 ml

Store at room temperature.

5. **1 M Tric-Cl in 1 L**

Tris base …………………………………………...........121.1 g

ddH$_2$O…………………………………………………..700 ml

Adjust to pH 7.5

Distilled water…………………………………………...….to make up 1000 ml

Autoclave. Store at room temperature.

6. **10 mM Tric-Cl in 1L**

   1 M Tris-Cl………………………………………….10 ml

   UP H$_2$O…………………………………………………990 ml

   Store at room temperature.

7. **10 mM Tric-Cl containing 2.5 mg/ml lysozyme**

   Filter sterilised lysozyme (25 mg/ml w/v in UP water)………100 µl

   10 mM Tris-Cl……………………………………………900 µl

   Add Lysozyme just prior to use.

8. **0.5 M EDTA, pH 8.0 in 500 ml**

   EDTA (disodium ethylenediaminetetraacetatic acid) …………93.05 g.

   ddH$_2$O………………………………………………….350 ml

   Adjust to pH 8.0

   Distilled water………………………………………………….to make up to 500 ml

   Autoclave. Store at room temperature.

9. **10 % SDS (Sodium dodecyl sulfate) in 10 ml**

   SDS……………………………………………………1 g

   UP H$_2$O…………………………………………………..10 ml

   Store at room temperature.

10. **50 mg/ml Proteinase K**

Proteinase K powder……………………………………..50 mg

UP H$_2$O…………………………………………………….. 1 ml

Store at -20 ^{o}C.

11. **Phage lyis buffer containing 50 mM EDTA, 50 mM Tris. Cl (pH 9.0, 1 % SDS) in 100 ml water**

1 M Tris. Cl (pH 8.0)…………………………………..5 ml of

0.5 M EDTA (pH 8)…………………………………..10 ml of

10 % SDS ……………………………………………10 ml (or use 1 g SDS powder) UP H$_2$O………………………………………….. to make up to 100 ml.

Store at room temperature.

12. **Phage lysis buffer with 0.5 mg of proteinase K** (proteinase K to be added just prior to use)

Phage lysis buffer…………………………………………9.9 ml

Proteinase K solution (50 mg/ml) …………………………100 µl

Add proteinase K just prior to use.

13. **5 x TBE (Tris borate EDTA) buffer (0.45 M Tris borate, 0.01 M EDTA) in 1L**

Tris base …………………………………………………..54 g add

Boric acid…………………………………………………..27.5 g and of

0.5 EDTA…………………………………………………..20 ml

UP H$_2$O……………………………………………………700 ml

Adjust pH to 8.0 and then bring to final volume of 1000 ml.

Store at room temperature.

14. 0.5 x TBE in 1000 ml

5 x TBE …………………………………………………100 ml

UP H$_2$O……………………………………………..900 ml

Store at room temperature

15. 10 x TE buffer (100 mM Tris. Cl, 10 mM EDTA) (pH 8.0) in 100 ml

1M Tris.Cl …………………………………………..10 ml of and

0.5 M EDTA……………………………………….2 ml

Distilled water…………………………………………….to make up to 100

ml

Autoclave. Store at room temperature

16. 1 x TE in 100 ml

10 x TE…………………………………………..10 ml

UP H$_2$O……………………………………………..90 ml

Store at room temperature.

17. 50 x TAE buffer (2 M Tris-acetic acid and 0.05 EDTA)

Tris Base……………………………………………242 g

UP H$_2$O……………………………………………..600 ml

Allow to dissolve and then add 57.1 ml Glacial Acetic Acid and 100 mL 0.5 M

EDTA mix and bring to a final volume of 1 L with UPH$_2$O. Store at room

temperature.

18. Bacterial lysis buffer containing 10 mM Tris-Cl (pH 8.0), 1 mM EDTA and

1 % SDS in 100 ml

1 M Tris-Cl……………………………………….1 ml

0.5 M EDTA …………………………………….20 µl

10 % SDS ……………………………………….10 ml

UP H$_2$O…………………………………………………......89.8 ml

Store a room temperature.

19. **Bacterial lysis buffer with 0.2 mg/ml proteinase K in 1 ml**

Bacterial lysis buffer…………………………..……….....1 ml

50 mg/ml proteinase K stock solution ………………......4 µl

Add proteinase K just prior to use.

20. **2 % w/v Plug agarose (Seeplaque® CTG agarose) in 0.5 x TBE in 1 ml**

Seeplaque® CTG agarose……………….…..……………… 2 mg

0.5 x TBE buffer……………………………………….…1 ml

Use straight away or keep warm at 55 ^{o}C.

21. **1 % w/v PFGE agarose (Pulsed-field certified megabase agarose) in 0.5 x TBE in 200 ml**

Pulsed-field certified megabase agarose……………..……2 g

0.5 x TBE buffer………………………………………200 ml

Heat to dissolve and cool to 50 ^{o}C before casting gel.

22. **1 % w/v RESponse Regular PCR Agarose gel in 1 x TAE in 100 ml**

RESponse Regular PCR Agarose………………………1 g of

1 x TAE………………………………………….....100 ml

Heat to dissolve and cool to about 50 ^{o}C before casting gel.

Solutions

1. **Mitomycin C (Fisher Scientific, Loughborough, UK) 300 µg/ml w/v**

Dissolve content of one 2 mg bottle of mitomycin C (2 mg of Mitomycin C and 48 mg of NaCl) in 6.67 ml of UP water. Protect from light by wrapping tube

with aluminum foil. Store at 4 ^{o}C. For prophage induction, use 10 µl/ml of stock

to give a final concentration of 3 µg/ml.

2. **Norfloxacin (Sigma-Aldrich, Dorset, UK) 300 µg/ml w/v**

   Norfloxacin powder……………………………………………..10 mg

   Absolute alcohol …………………………………………….33 ml.

   Cover tube with aluminum foil. Store at 4 ^{o}C. For prophage induction, use 10

   µl/ml of stock to give a final concentration of 3 µg/ml.

3. **2.5 mM dNTPs**

   100mM dATP…………………………………….…………20 ml

   100mM dCTP…………………………………….…………20 ml

   100mM dTTP …………………………………….…………20 ml

   100mM dGTP …………………………………….………...20 ml

   This solution will give a concentration of 25 mg/ml of dNTPS. Add 100 µl of the

   25 mg/ml stock to 900 µl to give a concentration of 2.5 mg/ml working solution.

   Use 1:10 in PCR to give 0.25 mM final concentration.

4. **2 mM dTTP**

   100 mM dTTP……………………………………………10 µl

   UP H$_2$O………………………………………………….50 µl

   This solution will give 20 mg/ml concentration of dTTP. Use 1 µl per 10 µl to

   obtain 2 mg/ml final concentration. Store at -20°C

5. **1M NaCl in 100 ml**

   NaCl………………………………………………….... 5.84 g

   Distilled water……………………………………………. to make up to 100 ml

   Autoclave. Store at room temperature.

6. **1 M KCl in 100 ml volume**

   KCl……………………………………………………………7.45 g

Distilled water ……………………………………….. to make up to 100 ml

Autoclave. Store at room temperature.

7. 1 M MgSO₄ in 100 ml volume

MgSO$_4$……………………………………………24.65 g

Distilled water………………………………………….to make up to 100 ml

 Autoclave and store at room temperature.

8. 1 M MgCl₂ in 100 ml volume

MgCl$_2$ …………………………………………….2.03 g

Distiiled water……………………………………. to make up to 100 ml

Autoclave and store at room temperature.
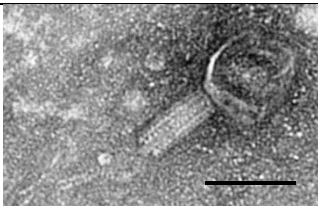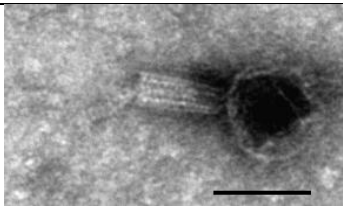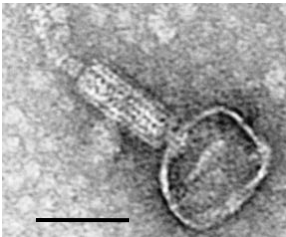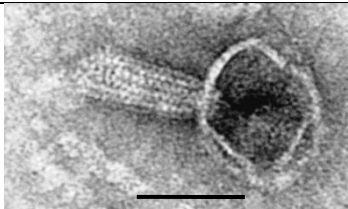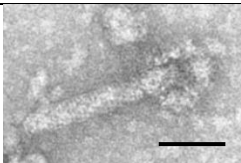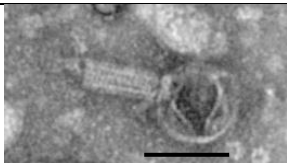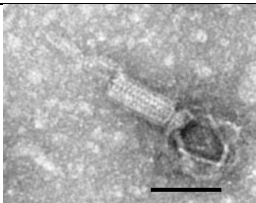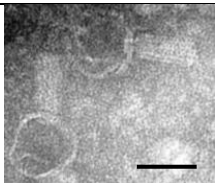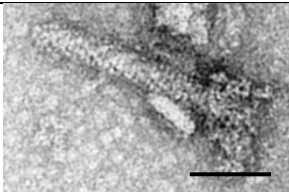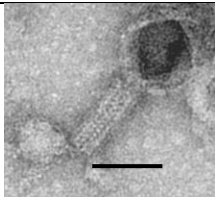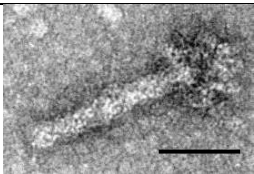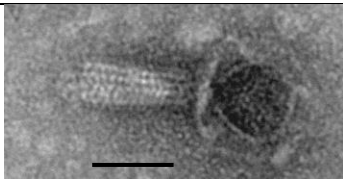
9. X-gal 40 mg/ml w/v in 2 ml volume
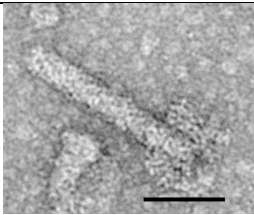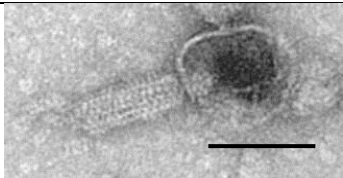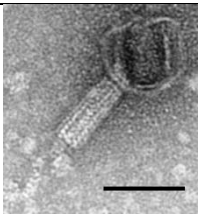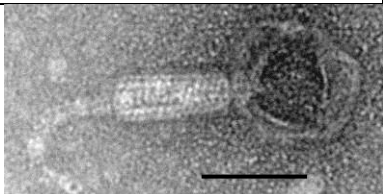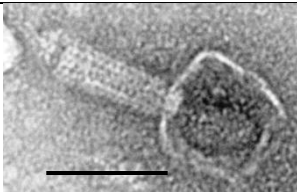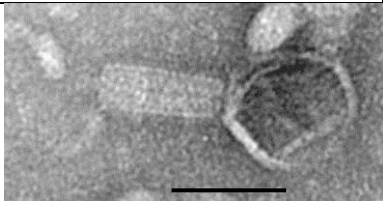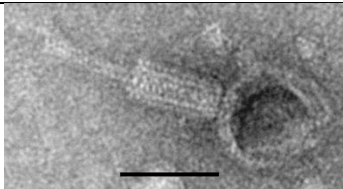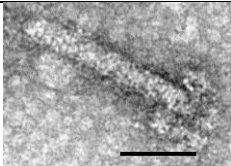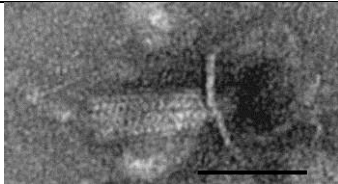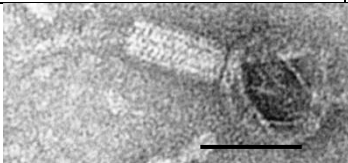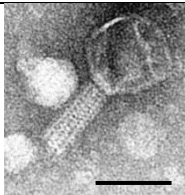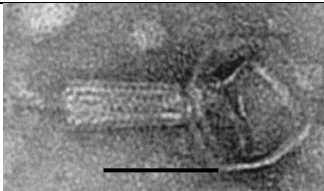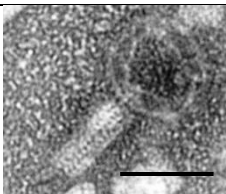
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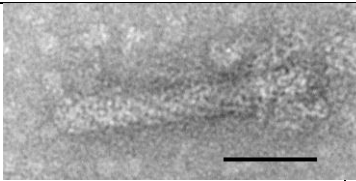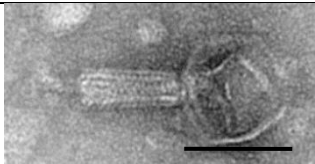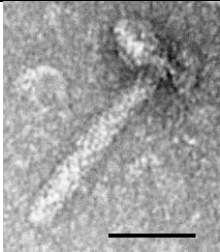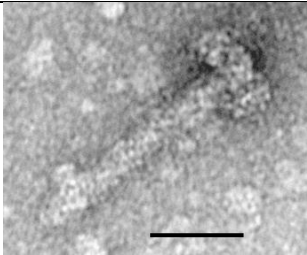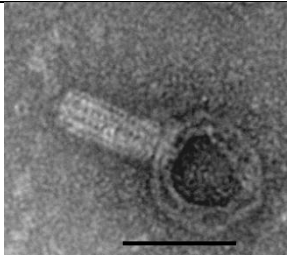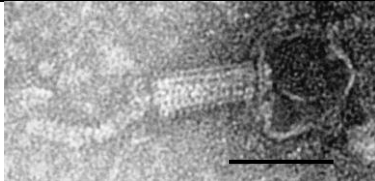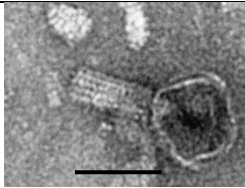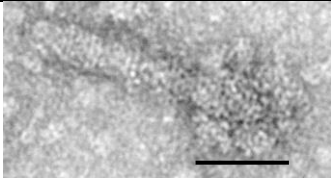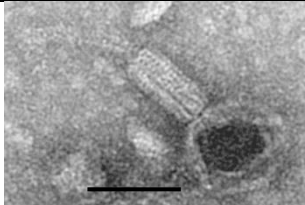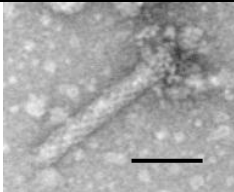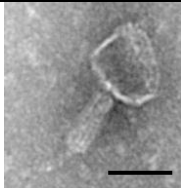
Dimethyl formamide…………………………………2 ml
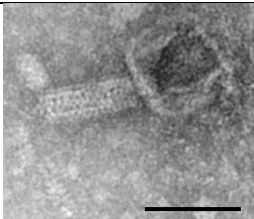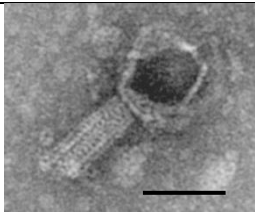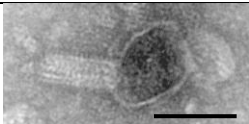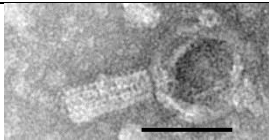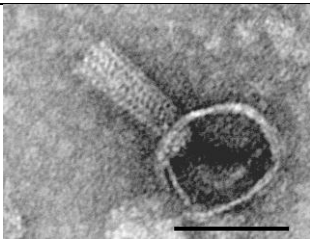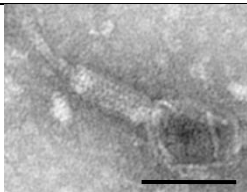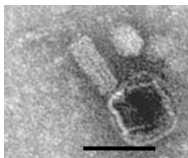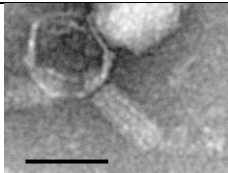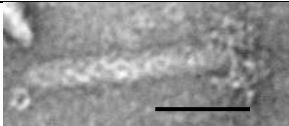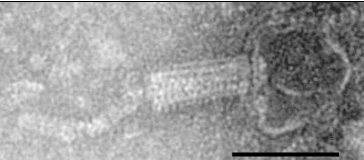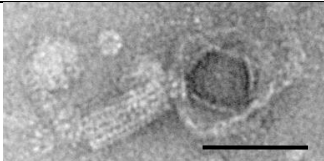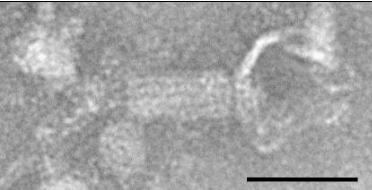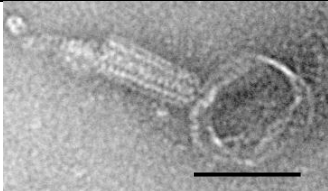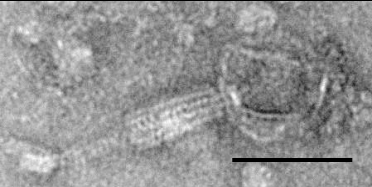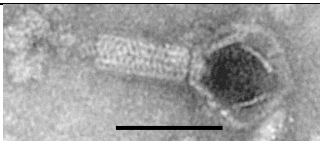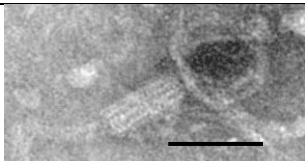
Dissolve in a glass ware. Do not autoclave. Dispense into eppendorf tubes, wrap

in foil to protect it from the light and store in -20°C freezer.
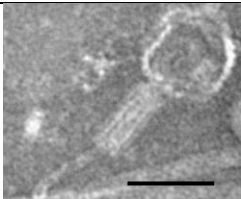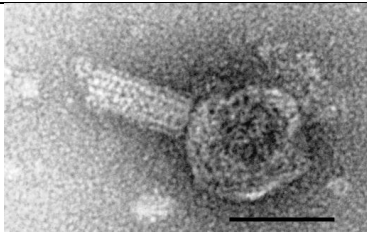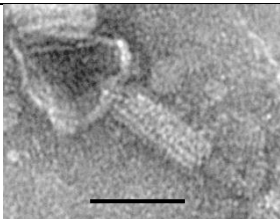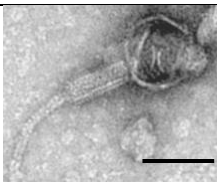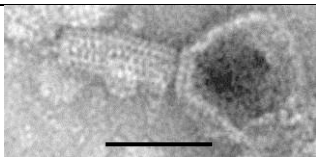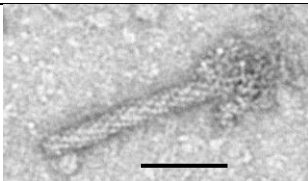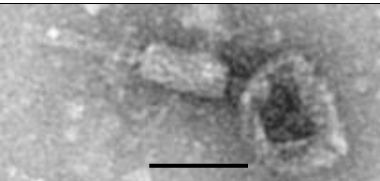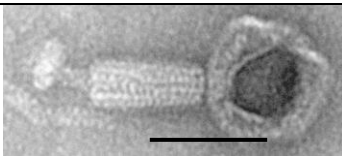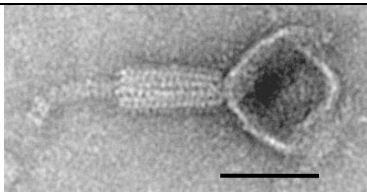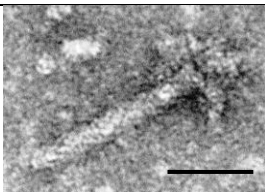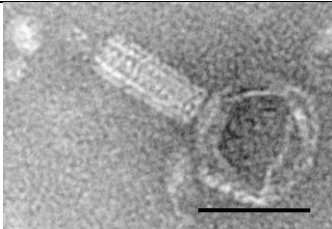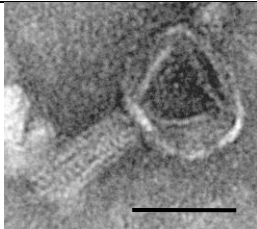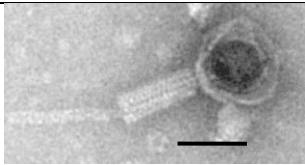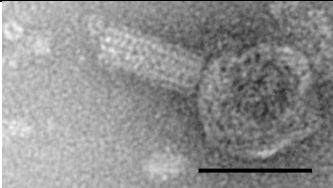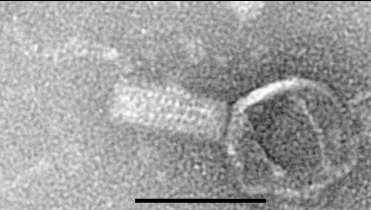
7.2 Appendix 2. Phage morphologies
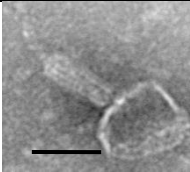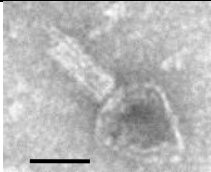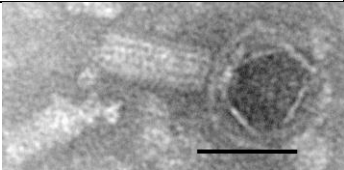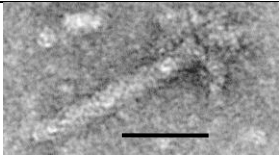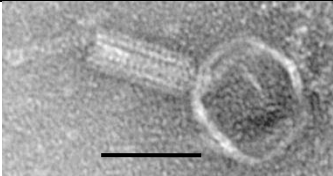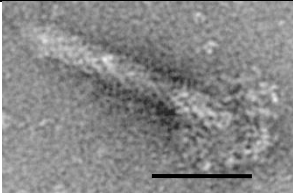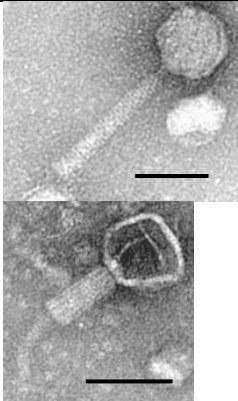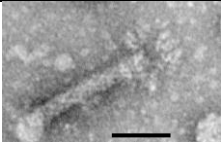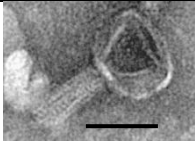
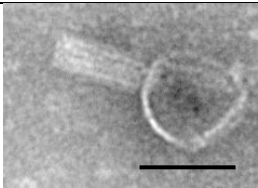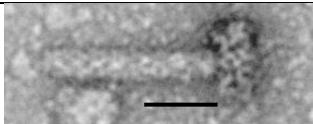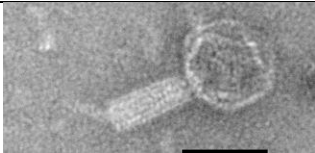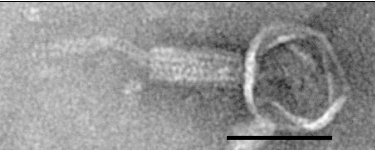| Isolates	MLVA	Pulsovars	Mitomycin C	Norfloxacin
14L	1	I		
52L	1	I		
38L	1	I		
2L	2	I		
12L	3	I		
87L	3	I		
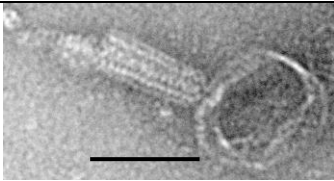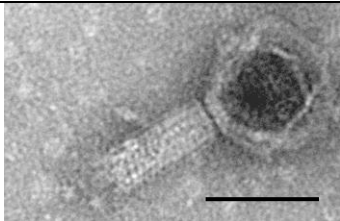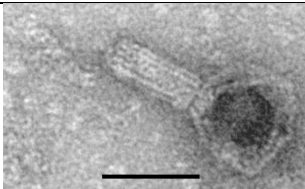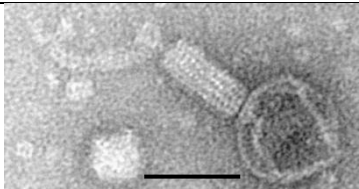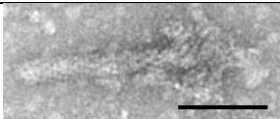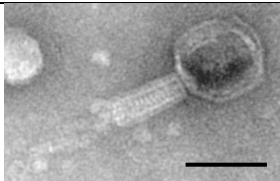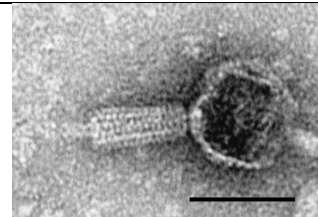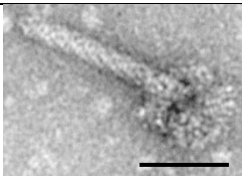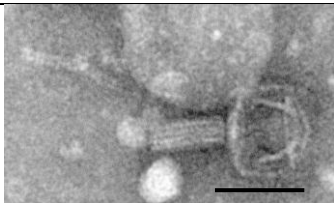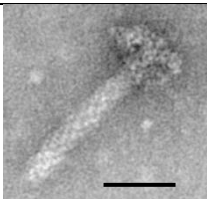
10L	3	I		
79L	3	I		
28L	3	I		
83L	4	I		
92L	4	I		
85L	4	I		
88L	4	I		
89L	4	I		
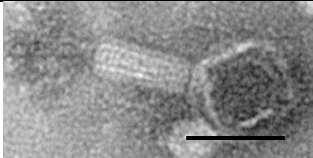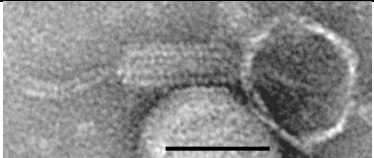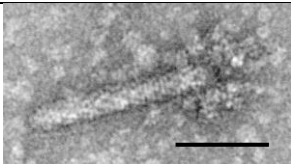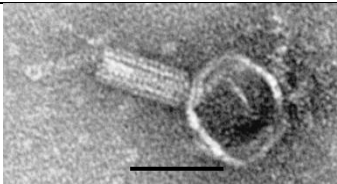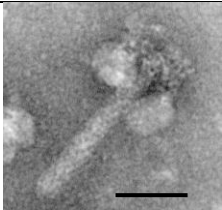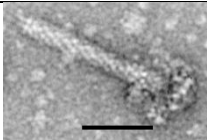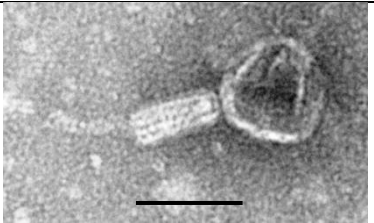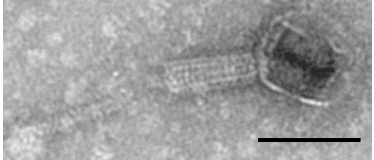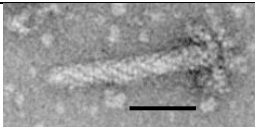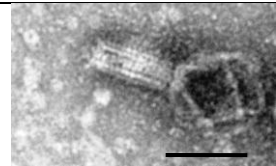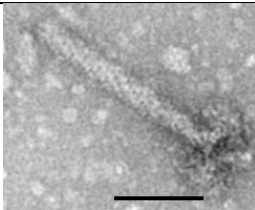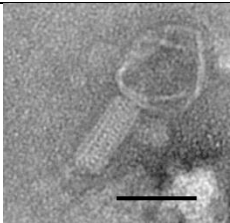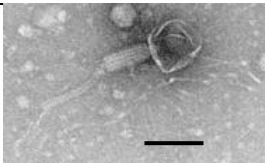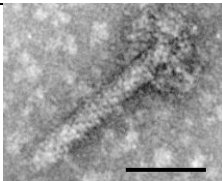
29L	4	I		
78L	4	I		
32L	5	I		
17L	6	I		
81L	6	I		
67L	7	I		
61L	7	I		
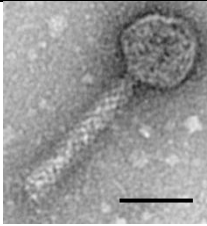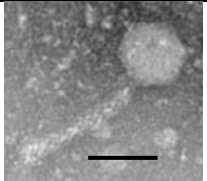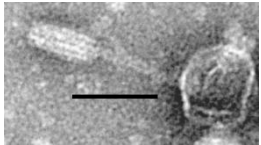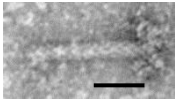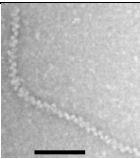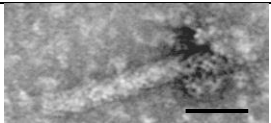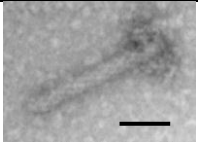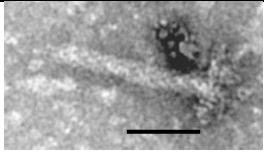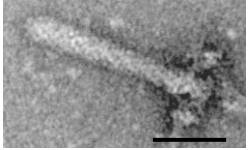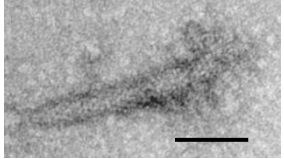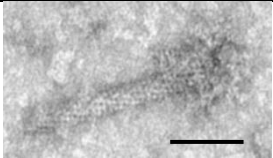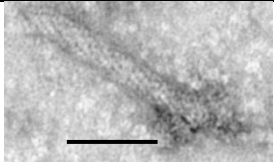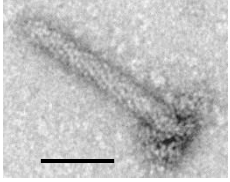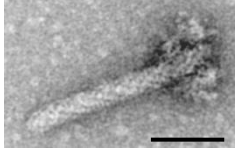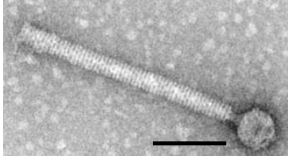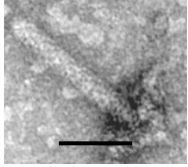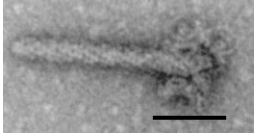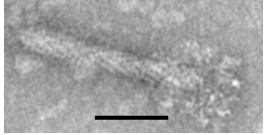
54L	7	I		
75L	7	I		
25L	7	I		
77L	7	I		
21L	7	I		
5L	8	I		
9L	8	I		
26L	9	I		
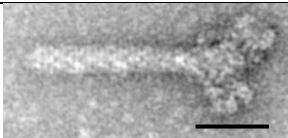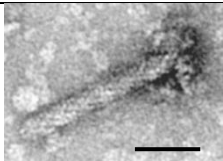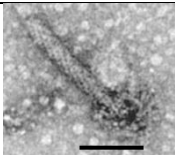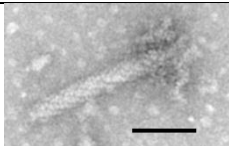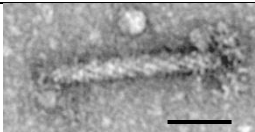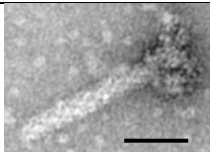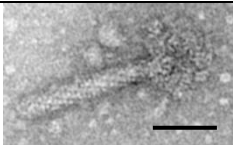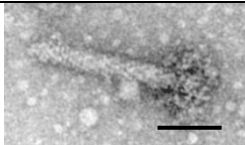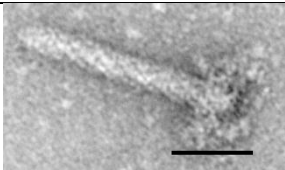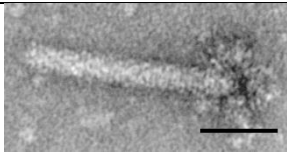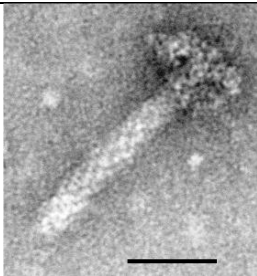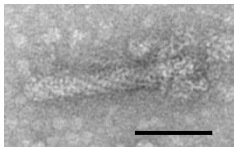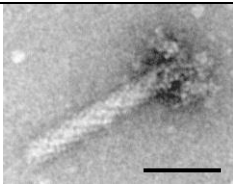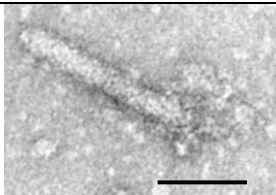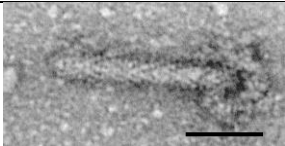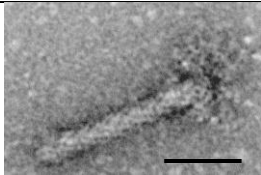
82L	9	I		
94L	10	I		
90L	11	I		
86L	11	I		
95L	11	I		
60L	12	I		
63L	12	I		
65L	12	I		
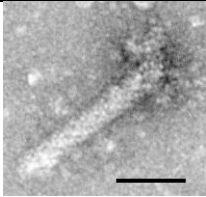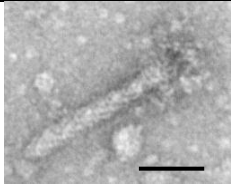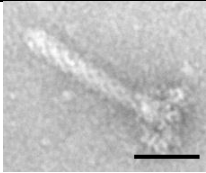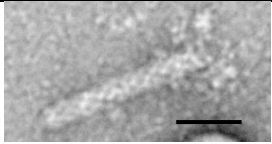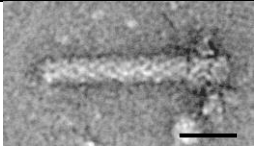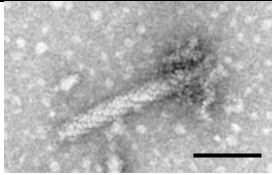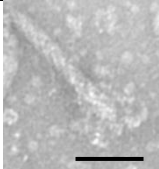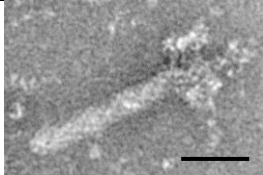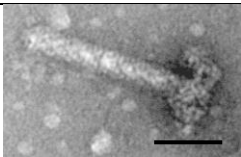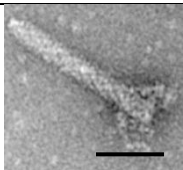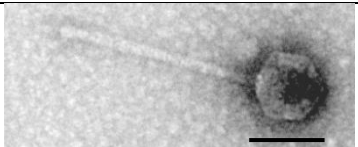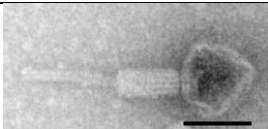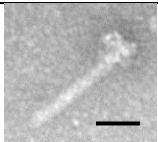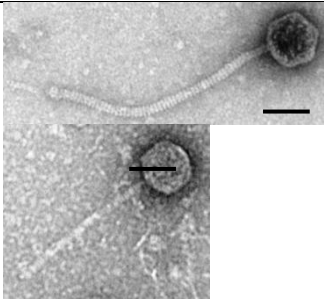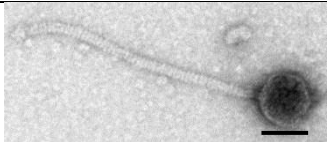
7L	12	I		
66L	12	I		
39L	12	I		
22L	13	I		
96L	13	I		
6L	13	I		
68L	13	I		
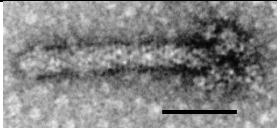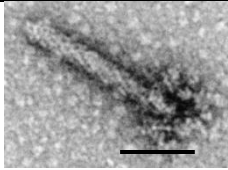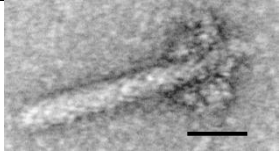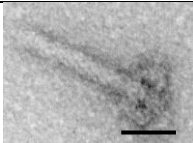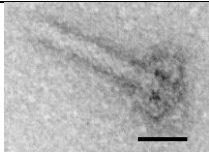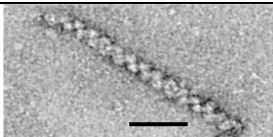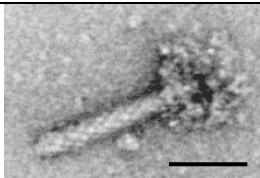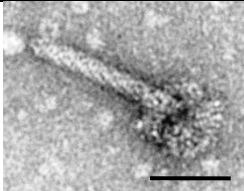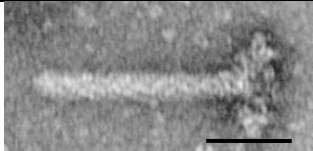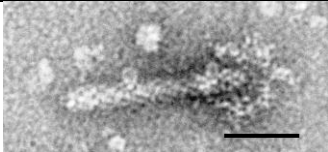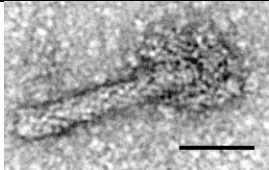
93L	13	I		
69L	13	I		
73L	13	I		
84L	13	I		
4L	13	I		
70L	13	I		
80L	13	I		
46L	13	I		

23L	14	IV		
47L	14	IV		
57L	14	IV		
31L	15	IV		
33L	15	IV		
34L	15	IV		
59L	15	IV		
20L	15	IV		

91L	15	IV		
3L	16	IV		
55L	16	IV		
1L	16	IV		
42L	16	IV		
15L	16	IV		
16L	16	IV		
19L	16	IV		

30L	16	IV		
35L	16	IV		
56L	16	IV		
50L	16	IV		
76L	16	IV		
18L	16	IV		
43L	16	IV		
44L	16	IV		

49L	17	IV		
71L	17	1		
58L	17	IV		
62L	17	IV		
64L	17	IV		
37L	18	III		
51L	18	II		
48L	18	III		

13L	19	IV		
53L	20	IV		
45L	20	IV		
40L	21	IV		
36L	22	V	No phage	No phage
41L	22	V		
72L	23	IV		

The table above shows diverse phage morphologies from mitomycin C and norfloxacin inductions of 91 *C. difficile* 027 isolates belonging to different subclades. Morphologies were identified using TEM. Bar ~70 nm based on the measurement of six phages in each sample.

7.3   Appendix 3. Prophage induction and OD_{550} values

Isolate	OD_{550} values			Pattern
	Before induction	**Mitomycin C**	**Norfloxacin**	
14L	1.146	0.730	1.670	B
52L	1.209	0.458	0.342	A
38L	0.972	1.56	0.645	C
2L	1.114	0.501	0.319	A
12L	1.232	1.80	0.861	C
87L	1.099	0.755	1.659	B
10L	1.245	0.874	1.642	B
79L	1.055	0.793	1.735	B
28L	1.142	0.521	0.312	A
83L	1.211	0.652	0.401	A
92L	1.118	0.715	1.702	B
85L	1.242	0.497	0.227	A
88L	1.108	0.341	0.379	A
89L	1.101	0.615	0.296	A
29L	1.003	0.923	1.732	B
78L	1.200	1.793	0.796	C
17L	1.019	0.501	0.378	A
32L	1.131	1.125	1.129	D
81L	1.142	0.857	1.721	B
67L	1.153	1.740	0.874	C
61L	1.1177	0.718	1.695	B
54L	1.182	0.471	0.235	A
75L	1.103	0.453	0.332	A
25L	1.013	0.368	0.218	A
77L	1.028	1.032	1.021	D
21L	1.035	0.798	1.592	B
5L	1.041	0.518	0.326	A
9L	1.024	0.874	1.722	B
26L	1.133	1.142	1.128	D
82L	1.217	1.221	1.215	D
94L	1.210	1.201	1.195	D
90L	1.251	1.248	1.283	D
86L	1.211	0.912	1.684	B
95L	1.162	0.523	0.339	A
60L	1.148	0.835	1.890	B
63L	1.202	1.197	1.216	D
65L	1.199	1.185	1.129	D
7L	1.137	1.134	1.128	D
66L	1.213	0.487	0.241	A

39L	1.154	0.871	1.748	B
22L	1.217	0.566	0.375	A
96L	1.122	1.593	0.861	C
6L	1.157	0.901	1.685	B
68L	1.008	0.988	1.022	D
93L	1.041	0.821	1.701	B
69L	1.107	1.056	1.011	D
73L	1.105	1.100	1.096	D
84L	1.027	1.122	1.036	D
4L	1.198	0.740	1.730	B
70L	1.031	1.044	1.027	D
80L	1.095	0.850	1.688	B
46L	1.113	0.739	1.809	B
23L	1.290	1.220	1.301	D
47L	1.103	0.810	1.811	B
57L	1.049	0.722	1.753	B
31L	1.067	0.710	1.800	B
33L	1.022	1.008	1.053	D
34L	1.002	0.694	1.870	B
59L	1.084	0.733	1.893	B
20L	1.028	0.827	1.920	B
91L	1.079	0.479	0.204	A
3L	1.189	0.513	0.320	A
55L	1.103	0.447	0.264	A
1L	1.092	0.610	0.340	A
42L	1.105	0.311	0.275	A
15L	1.014	0.501	0.331	A
16L	1.038	0.573	1.694	B
19L	1.201	0.437	0.320	A
30L	1.089	1.075	1.062	D
35L	1.193	0.562	0.314	A
56L	1.022	1.017	1.027	D
50L	1.136	0.553	0.377	A
76L	1.210	0.490	0.302	A
18L	1.026	0.521	0.331	A
43L	1.205	0.511	0.308	A
44L	1.033	1.045	1.068	D
49L	1.127	0.532	0.211	A
71L	1.214	1.220	1.275	D
58L	1.003	0.427	0.226	A
62L	1.022	1.053	1.031	D
64L	1.027	0.497	0.225	A
37L	0.98	0.438	0.220	A
51L	1.238	0.713	1.920	B
48L	1.117	0.350	0.209	A
13L	1.004	1.022	1.052	D

53L	1.132	1.173	1.129	D
45L	1.002	0.482	0.318	A
40L	1.031	1.048	1.026	D
36L	1.049	0.502	0.238	A
41L	1.046	0.742	1.840	B
72L	1.238	0.425	0.337	A

The table above shows the growth responses (OD_{550} measurements of *C. difficile* 027 isolates before and after mitomycin C and norfloxacin inductions at 3μg/ml concentration.

7.4 Appendix 4. Capsid nucleotide sequences of *C. difficile* strains

C. difficile 027 isolates sequences

>80L

TATTCAAATGTTTTAGCACAAGCATATCCTTATACTTTAAACTTCGGGGATTTGTATGCAACAGAAAATAATGGAAGA
TATAGATGGACGGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGGGTAGATTCAAACAGAGATACA
ATAGCAGTAGCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGTATTAACTAATCAAAGGAAATGGTCAACATTG
GTTCATCCAGCAGATATAAACCAAACTAATTATGTGGCTTCAATAGGTAATATAACAAAAGTATATAATGAGGAACAA
AAGTTTCCAGAGATGGATGCTTACTG

>**73L**

TATTCAAATGTTTTAGCACAAGCATATCCTTATACTTTAAACTTCGGGGATTTGTATGCAACAGAAAATAATGGAAGA
TATAGATGGACGGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGGGTAGATTCAAACAGAGATACA
ATAGCAGTAGCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGTATTAACTAATCAAAGGAAATGGTCAACATTG
GTTCATCCAGCAGATATAAACCAAACTAATTATGTGGCTTCAATAGGTAATATAACAAAAGTATATAATGAGGAACAA
AAGTTTCCAGAGATGGATGCTTACTG

>**90L**

TATTCAAATGTTTTAGCACAAGCATATCCTTATACTTTAAACTTCGGGGATTTGTATGCAACAGAAAATAATGGAAGA
TATAGATGGACGGGTTCTAAAACAATAGAAATACCAACTATATCTACAACTGGAAGGGTAGATTCAAACAGAGATACA
ATAGCAGTAGCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGTATTAACTAATCAAAGGAAATGGTCAACATTG
GTTCATCCAGCAGATATAAACCAAACTAATTATGTGGCTTCAATAGGTAATATAACAAAAGTATATAATGAGGAACAA
AAGTTTCCAGAGATGGATGCTTACTG

>91L

TATTCAAATGTTTTAGCACAAGCATATCCTTATACTTTAAACTTCGGGGATTTGTATGCAACAGAAAATAATGGAAGA
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>68L

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>82L

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>96L

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ATAGCAGTAGCTCAAAGAAACTATGATAATGCTTGGGAACCTAAGGTATTAACTAATCAAAGGAAATGGTCAACATTG

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>**16L**

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>**45L**

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>**41L**

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>**CD196**

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>**CDR20291**

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AAGTTTCCAGAGATGGATGCTTACT

Other *C. difficile* ribotypes sequences

>**014**

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209

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>**CD630 (CD012)**

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>**CD015**

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>**CD001**

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>**002**

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CAGAGATGGATGCTTATTGA

>005

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TAGCTCAAAGAAACTATGATAATGCTTGGGAATCTAAAACATTAACTAACCAAAGAAAATGGTCTACATTGGTTCATC

CAGCAGATATTGACCAAACTAACTATGTAGCATCAATAGGAAATATAACTCAAGTATACAATGAGGAACAAAAATTA

7.5 Appendix 5. Holin nucleotide sequences of *C. difficile* strains

C. difficile 027 isolates sequences

>80L

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GTTGGGGAGTTGCAATAGGTATAAATCAAAC

>52L

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GTTGGGGAGTTGCAATAGGTATAAATCAAAC

>16L

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>001

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>002

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>014

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GTTGGGGAGTTGCAATAGGTGTAAATCAAAC

213

>015

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GTTGGGGTATATCAATAGGTATAAACCAAAC

>020

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GTTGGGGAGTTGCAATAGGTGTAAATCAAAC

>078

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TGTTGTGCCAGAAGATGATTATGTAATTGAAATTGGAAAGGCAGATATAAAAAAAGAGGGTACTGATGTTACAGTAAT

CACATATGGAAGAATG

214

7.6 Appendix 6. Portal protein nucleotide sequences of *C. difficile* strains

C. difficile 027 isolates sequences

>16L

GGGCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCCACCGTGGAGTAGCACTTAAATTTTTATATT
CACTACTTGATTTAAAATGTTCCAAGACTGAAAAGAAGTTTAAAAAAGCAATTAGAGAGCTTTTATGGTTTGTGTGTG
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>90L

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>41L

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TGGGTTGATGATGA

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>73L

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CAAAAGGTGTTAGAAGACGACAAGAATTTAAGTGCTCCA

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>91L

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TTATTGGCATTACAAATAATGACTTTAAAATACCCAAAATTAAAAGAGGATTTGAAATCATATAGAGAAGAGATGGCA

CAAAAGGTGTTAGAAGACGACAAGAATTTAAGTGCTCCA

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TCACTACTTGATTTAAAATGTTCCAAGACTGAAAAGAAGTTTAAAAAAGCAATTAGAGAGCTTTTATGGTTTGTGTGT

GAGTATTTAAAGATAAGTGGTAGTAAGAGCTATGATTATAAGTCAGTTCAAATAACATTTAATCATTCTATGATAATA

AATGAGTCTGAAAAAATAGATATGGCTTCTAAATCAATTGGAATTATATCAGATGAAACTATTGTTAGTAACCATCCT

TGGGTTGATGATGA

Other ribotypes sequences

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>CD020

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078

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>CD630

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TCTTTAAATACTCACACACAAACCATAAAAGCTCTCTAATTGCTTTTTTAAACTTCTTTTCAGTCTTAGAACATTTTA

AGTCAAGTAGTGAATATAAAAATTTAAGTGCTACACCCG

7.7 Appendix 7. Capsid protein sequences

C. difficile 027 capsid protein sequences

>16L

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VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>41L

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VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>45L

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>73L

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VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>80L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>82L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>84L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>90L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>91L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>96L

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

Other *C. difficile* ribotypes and bacterial capsid protein sequences

>Bacillus-phage-250

MATLNYATQYQEVLVQKFSQGAAFGALYATPNNDVVKWTGPKTIQIPRIKVGGYTDVNRDVVGNYTRRVDNSFEPKTL

GHDREFRTLVDPADIDETNMAVSIANITRVFNDEEAIPEHDKYMASKLYAEFTGAGKTADTTALTPESFLEVFDNMML

EMDEAEVPQTGRIMYITPAVKKIVKAAKELQRTLEISGTTEKAVNRGVYSLDDVTIITVPSSRMKTAYNFTNGAVPDA

TAKQINIILIHPLSVVAPQKYEFVDLDTPSAATGGKYLYYERKYWDVFILGAKVDGVKFNITSA

>Bacillus-phage-IEBH

MATLNYAAQYQEALVQKFAQGAAFGALYNTPNNNIVKWTGPKTIQIPSIKVGGYTDVNRDVVGNYTRRVDNSFEPKTL

GHDREFRTLVDPVDIDETNMAVSIANITRVFLNEESIPEHDKYMASKLYSEFTGAGKTADATVLTAANILSVFDQMML

EQDEAEVPQDGRLLYVTPAVKKLLKEAEQIQRTLDIKGAGENAVNRNVYSLDDVTIVTVPSSRMKTAYNFTNGAVPDA

AAKQINMILVHPLAVVSPQKYEFVDLDTPSAATGGKYLYYERKYWDVFILGAKVAGVKFNITTA

>CD196

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>CDR20291

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>QCD-66c26

YSNVLAQAYPYTLNFGDLYATENNGRYRWTGSKTIEIPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTL

VHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>*Clostridium-difficile*-001

YSNVLAQAYPYTLNFGDLYYSNGLAQAYPYTLNFGDLYATENPGRYRWTGSKSIEIPTISTTLRVDXNRDTIAVAQRN

YDNAWEPKVLTNQRKWSTLVQTADINQTKHVASIGNITKVYNEEQKFPEMDAYR

>*Clostridium-difficile*-002

YSNVLAQAYPYTLNFGDLYADVACSRPPWRPREFDPLENGQVYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIE

IPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTLVHPADINQTNYVASIGNITKVYNEEQKFPEMDAYX

>*Clostridium-difficile*-005

YSNVLAQAYPYTLNFGDLYADVAGSRPPWRPREFDPLNNGKVYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIE

IPTISTTGRVDSNRDTIAVAQRNYDNAWESKTLTNQRKWSTLVHPADIDQTNYVASIGNITQVYNEEQKL

>*Clostridium-difficile*-014

YSNVLAQAYPYTLNFGDLYPIVSRITIHWPSFYNVVTGKTLALPNLIALQHIPLSPAGVIAKRPAPIALPNSCAAXMA

NGRALXRRIKRGGCGGYAQRDRYTCQRPSARSFRFLPFLSRHVRRLSPSSSKSGX

>*Clostridium-difficile*-015

YSNVLAQAYPYTLNFGDLYADVACSRPPWRPREFDPLKYGKEYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIE

IPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTLVHPADINQTNYVASIGNITKVYNEEQKFPEMDAYRF

VX

>*Clostridium-difficile*-020

YSNVLAQAYPYTLNFGDLYADVACSRPPWRPREFDPLKDAKEYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIE

IPTISTTGRVDSNRDTIAVAQRNYDNAWEPKVLTNQRKWSTLVHPADINQTNYVASIGNITKVYNEEQKFPEMDAYR

>*Clostridium-difficile*-630

YSNVLAQAYPYTLNFGDLYMAALNYAKEYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIEIPTISTTGRVDSNR

DTIAVAQRNYDNAWEPKVLTNQRKWSTLVHPADINQTNYVASIGNITKVYNEEQKFPEMDAYCISKIYADWTTLGNTA

DTTVLTTTNVLEVFDKLMEKMTEARVPENGRILYVTPVVNTLIKNAKEIQRTVNIKDAGTSLNRQTTDIDTVKIIKVP

SNLMKTAYDFTTGWKVGAGAKQIFMSLVHPSAIITPVSYQFSKLDEPTAVTEGKYFYFEESFEDVFILNKKADAIQFV

VEGAGA

>*Clostridium-difficile*-phage-phiC2

YSNVLAQAYPYTLNFGDLYMAALNYAKEYSNVLAQAYPYTLNFGDLYATPNNGRYRWTGSKTIEIPTISTTGRVDSNR

DTIAVAQRNYDNAWEPKVLTNQRKWSTLVHPADINQTNYVASIGNITKVYNEEQKFPEMDAYCISKIYADWTALGNTA

DTTVLTTTNVLEVFDKLMEKMTEARVPENGRILYVTPVVNTLIKNAKEIQRTVNIKDAGTSLNRQTTDIDTVKIIKVP

SNLMKTAYDFTTGWKVGAGAKQIFMSLVHPSAIITPVSYQFSKLDEPTAVTEGKYFYFEESFEDVFILNKKADAIQFV

VEGAGA

>*Clostridium-difficile*-phage-phiCD119

YSNVLAQAYPMANTLAYGQVLQQGLDKQATQELLTGWMDSNAKQIKYEGGKEVKIGKLSTDGLGDYSRGSANAYVGGD

VKFEYETKTMTQDRGRKFTLDAMDVDETNFLVTATTVMGEFQRLKVIPEIDAYRLSRLATIAIGIKGDTNVEYSYSVN

SSTIINKIKTGIKIIRENGYNGPLVCHLTYDSMFAIEEKVLEKLTAVTFAQGGIQTQVPSIDGCALIKTPQNRMYSSI

LLNDGTTSNQTAGGYLKGTKALDTNFIIAPVDVPLAITKQDKMRIFDPETNQTANAWSMDYRRYHDLWVTDNKANSVY

ANFKDAKPVG

>*Clostridium-butyricum*-BL5262

YSNVLAQAYPPNLNYATQYSQALAQQFPYVLYFGALYATPNNGRYKVTGAKTIEIPVLSTTGRVDGDRDGIGTPNRNY

NNEWEPKVLTNHREWDTLVHPLDIDQTNQVASIGNITQVYNEEQKFPEMDAYTISKVYSDWIAQSKTPIKIALTTANV

LVQFDAMMQKMDEARVPVQGRVLYVTPGVKTLIKNAEAIQRQLDVQNGNGAVNRNVSRLDEVEIVSVPSDLMKTVYDF

TKGWKAGVGAKQVQMLLIHPVAVITPVTYSFSSLDTPSAKTHGKYYYYEESFEDVFILNKKADAIQFLQEADA

>*Clostridium-perfringens*-ATCC-13124

YSNVLAQAYPMAVYSYAEQFERQLQQKYARELTSYALEQSNPQVKFINAQTIKLPNITVSGYKDHNRSAMGFNTGSMS

NDWEPKKLSHDRDIEFAIDPMDVDETNLTLEMANIQNVFETEQAIPEKDSYRYSKLYAEAKTYKANGAVIDNTVLTTA

NILDWFDTQMEKMDDLGVPSEGRILYVTPAINKLLKNAEGLTRTINSDKNTGKVDRRVYSLDDVTITKVPSARMKTKY

DFTNGCVPAGDAKQINIILIHPSCQVTRSKYSYIKVFTPGTDSRTADKYVFQNRSYGDTFLIKNKACGIAINAESEG

>*Clostridium-perfringens*-B-str.-ATCC-3626

YSNVLAQAYPMAVYSYAEQFERELQQKYARELTSYDLEQSNPQVKFINAQTIKLPNITVSGYKDHNRGNMGFNTGTIS

NEWEPKKLAHDRDIEFALDPMDIDETNLTLEMANVQNTFETEQAIPERDSYRYSKLYAEAKTYKSNGAVIDNTVLTTA

NVLDWFDEKMEKMDDEGVPSEGRILYVTPAMNKIIKNAQNIQRSLDVNSNNGNVDRRVYSLDDVTIKKVPSARMKTKY

DFTNGCVPAGDAKQIHMILIHPSCQVTRRKYAYMKLFTPGTDSRTADNYVYQTREYGDTFLIKNKACGIAINAEAEG

>*Lactobacillus-gasseri*-JV-V03

MTINYAEKYQAAVQQAFYDGHLYSAELWNSPSNSIIKFDGAKHIKVPRLEITSGRKDRQRRTITTPVANYSNDWDSYE

LKNERYWSTLVDPSDIDETNMVVSLANITKQFNLDSKMPEKDRYMFSHLYSGKEAAHDGGITTNTLDEKNILPAFDNM

MLDFDEARIPSTNRILYVTPKTNAILKRAEAMNRALTLKDPNNIQRTVYSLDDVTIRVVPSDLMQTAYDFSDGSKIID

TAKQIEMFLIYNGVQIAPEKYSFVGFDQPSAATSGNYLYYEQSYDDVLLLNTKTKGIQFVVSDKPKKDQEQSGQEHKD

QDAKPTAESTLEEIKAYLDKNHIDYTGKTKKDELLTLV

>*Lactobacillus-phage*-KC5a

MTINYAEKYQAAVQQAFYDGHLYSAELWNSPSNSIIKFDGAKHIKVPRLEITSGRKDRQRRTITTPVANYSNDWDSYE

LKNERYWSTLVDPSDIDETNMVVSLANITKQFNLDSKMPEKDRYMFSHLYSGKEAAHDGGITTNTLDEKNILPAFDNM

MLDFDEARIPSTNRILYVTPKTNAILKRAEAMNRALTLKDPNNIQRTVYSLDDVTIRVVPSDLMQTAYDFSDGSKIID

TAKQIEMFLIYNGVQIAPEKYSFVGFDQPSAATSGNYLYYEQSYDDVLLLNTKTKGIQFVVSDKPKKDQEQSGQDAKP

TAESTLEEIKAYLDKNHIDYTGKTKKDELLALVK

>*E. coli*-phage

MKTPTIPTLLGPDGMTSLREYAGYHGGGSGFGGQLRSWNPPSESVDAALLPNFTRGNARADDLVRNNGYAANAIQLHQ

DHIVGSFFRLSHRPSWRYLGIGEEEARAFSREVEAAWKEFAEDDCCCIDVERKRTFTMMIREGVAMHAFNGELFVQAT

WDTSSSRLFRTQFRMVSPKRISNPNNTGDSRNCRAGVQINDSGAALGYYVSEDGYPGWMPQKWTWIPRELPGGRASFI

HVFEPVEDGQTRGANVFYSVMEQMKMLDTLQNTQLQSAIVKAMYAATIESELDTQSAMDFILGANSQEQRERLTGWIG

EIAAYYAAAPVRLGGAKVPHLMPGDSLNLQTAQDTDNGYSVFEQSLLRYIAAGLGVSYEQLSRNYAQMSYSTARASAN

ESWAYFMGRRKFVASRQASQMFLCWLEEAIVRRVVTLPSKARFSFQEARSAWGNCDWIGSGRMAIDGLKEVQEAVMLI

EAGLSTYEKECAKRGDDYQEIFAQQVRETMERRAAGLKPPAWAAAAFESGLRQSTEEEKSDSRAA
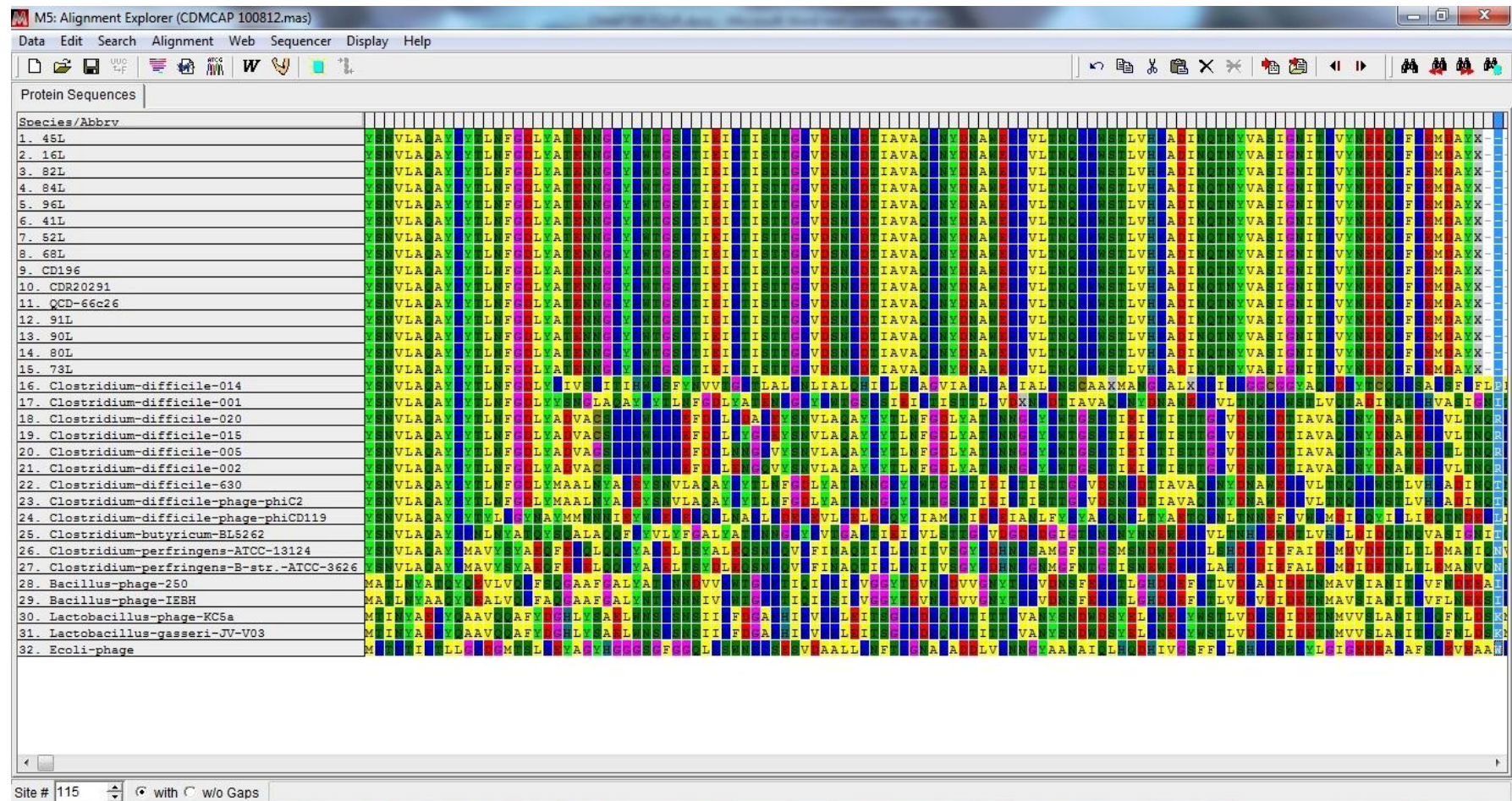
Figure 7.1 Multiple alignment of the phage capsid genes showing amino acids in region 1-115

The alignment involved 32 amino acid sequences of 12 (84L, 16L, 96L, 82L, 52L, 68L, 91L, 90L, 80L, 73L, 45L and 41L) representative isolates of the ribotype 027 subclades and six other ribotypes (ribotypes 014, 005, 002, 020, 015 and 001). Four other sequences including CD196, CDR20291 and QCD-66c26 (ribotype 027) and CD630 (ribotype 012) were obtained from *in silico* PCR. Other sequences were obtained from NCBI searches. Alignment was performed in MEGA5.01

Figure 7.2 Multiple alignment of the phage capsid genes showing amino acids in region 115-229

223
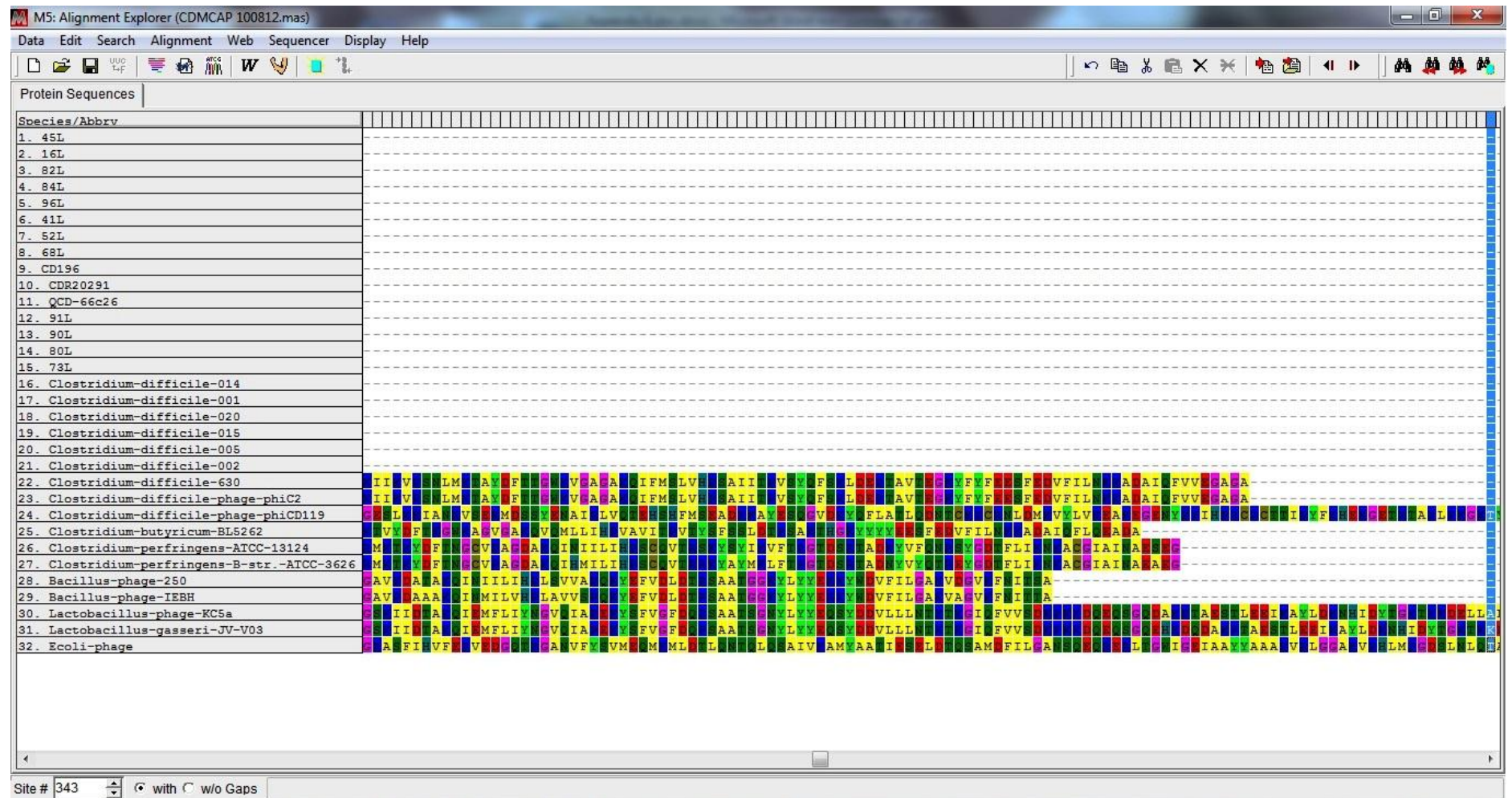
Figure 7.3 Multiple alignment of the phage capsid genes showing amino acids in region 229-343

Figure 7.4 Multiple alignment of the phage capsid genes showing amino acids in region 343-457
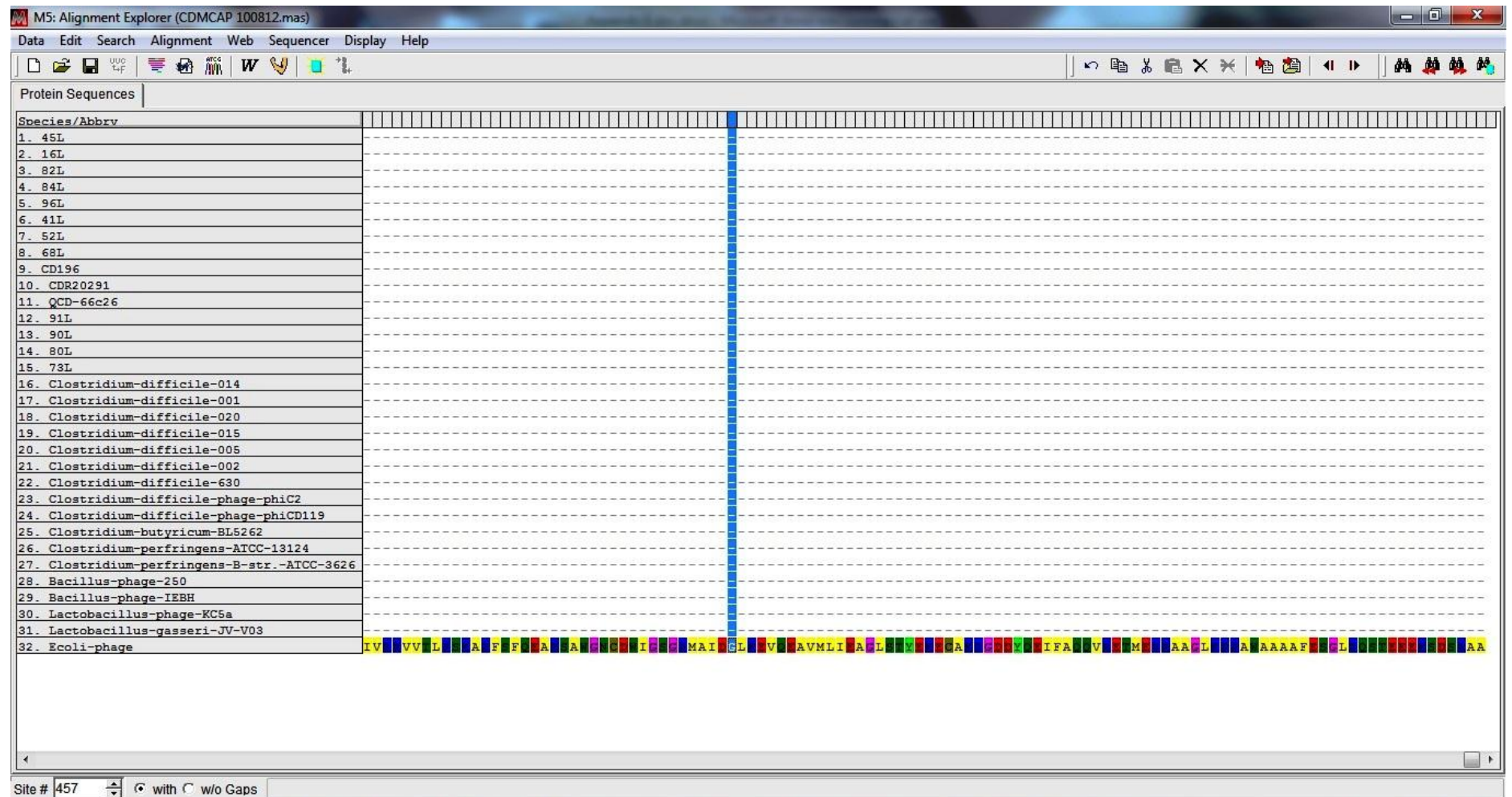
Figure 7.5 Multiple alignment of the phage capsid genes showing amino acids in region 457-533 (from the blue line)

7.8 Appendix 8. Holin protein sequences

C. difficile 027 holin protein sequences

>CD630

LVAALYVIGAGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPSAVLQGVICWGISIGIN

>002

LVAALYVLGVGFKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>013

LVAALSIIGKGCKKYKQLDNKYIPIVLLVLGIGFSIWMLGLSPVAVLQGVICWGISIGIN

>014

LVAALYVIGAGCKKYKQLDDKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGVNQT

>015

LVAALSIIGKGCKKYKQLDNKYIPIVLLVLGIGFSIWMLGLSPVAVLQGVICWGISIGINQT

>020

LVAALYVIGAGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGVNQT

>001

LVAALSIIGKGCKKYKQLDNKYIPIILLVLGIGFSIWMLGLSPNAVLQGVICWGISIGINQT

>107

LVAALYVIGAGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGVNQX

>CD196NC

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>R20291NC

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>73L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>41L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>96L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>90L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>68L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>16L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

96L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>52L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>80L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>91L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>45L

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

Other *C. difficile* ribotypes and bacterial holin protein sequences

>CD-phage phiC2

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLNPVAVLQGVICWGVAIGINQT

>phiCD27

LVAALSIIGKGCKKYKQLDNKYIPVVLLILGIGFSIWMLGLSPVAVLQGVICWGISIGINQT

>QCD-63q42

MDNLISFIPEQLLILVAALSIIGKGCKKYKQLDNKYIPIILLVLGIGFSIWMLGFNPSSILQGILCWGVAIGANQVYK

QLKEENK

>CD phage phi119

MDNLISFIPEQLLILVAALYVIGAGCKKYKQLDNKYIPVVLLALGIGFSIWMLGLNADSVLQGVICWGVAIGINQTYK

QLKEENK

>*C-perfringens*-ATCC-3626

MDNIIKFVPEQLLILVAALYIIGYFLKKTPKVLNWTIPWILMFLGIGFSISIMGLNATSILQGIICSFSAVATNQFIK

QTINK

>Clostridium-botulinum-E3-str-Alaska-E43

MDFMTYISQNALILIPALYIVGMVIRGTESIPNKFIPFILLIIGIVGAMFLLGFNINGAIQGILVTGVTVYTNQLFKQ

YNKNE

>Bacillus-sp-B14905

MEFLYDYIIEQALIVVPVLLVIGQALKNTPKMQDWLIPYILLVFGITFTIGVMGITMQSIVQGVLVSGAAVFSNQLYK

QYSHKEGNGK

>B*acillus-cellulosilyticus*-DSM-2522

MEFLYDYIIEQAYIVVPVGLVIGQALKNTPKMQDWLIPYILGVFGITFTIGVMGITMQSIVQGVLVSGAAVFSNQLYK

QYSHKEGNGK

>*Clostridium-perfringens*-E-str-JGS1987

MENIIKFVPEQLLILVAALYVIGMFLKKTPKVVDWSIPWILVVLGVGFSVAIMGINPTSILQGVICAFGAIATNQLVK

QTVNK

>*Clostridium-perfringens*-CPE-str-F4969

MENIIKFVPEQLLILVAALYIIGYFLKKTPKVLNWTIPWMLMFLGIGFSISIMGLNANSILQGIICSFSAVATNQFVK

QTIKK

>*Clostridium-butyricum*-5521

MDVSQYITQNALILIPVLYIIGMIIKNTDKISDKYIPLILLVFGIAGSMGIIGINANAIIQGVLVTGATVYTNQLIKQ

TGKDK

>*Clostridium-botulinum*-B-str-Eklund-17B

MDFMTYISQNALILIPALYIVGMIIRGTESIPNKFIPFILLIIGIIGAMFLLGFNINGAIQGILVTGVTVYTNQLFKQ

YNKNE

>*Clostridium-botulinum*-E1-str-BoNT-E-Beluga

MDFMTYISQNALILIPALYIVGMIIRGTESIPNKFIPFILLIIGIIGAMFLLGFNINGAIQGILVTGVTVYTNQLFKQ

YNKKE

>*E. coli* phage-lambda

MKMPEKHDLLAAILAAKEQGIGAILAFAMAYLRGRYNGGAFTKTVIDATMCAIIAWFIRDLLDFAGLSSNLAYITSVF

IGYIGTDSIGSLIKRFAAKKAGVEDGRNQ
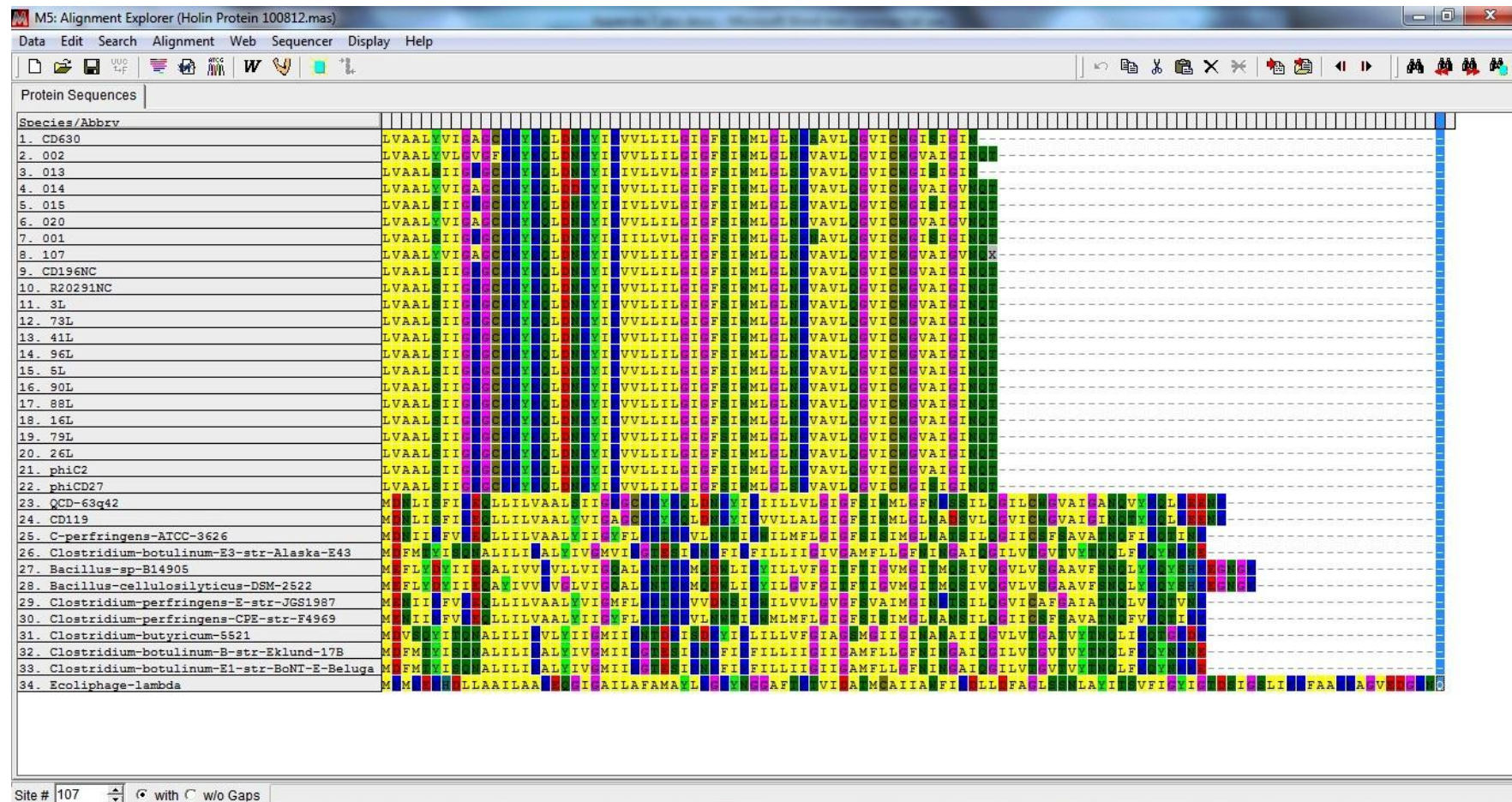
Figure 7.6 Multiple alignment of the holin gene sequences showing the 1-107 amino acids

The alignment involved 34 amino acid sequences of 10 (80L, 52L, 16L, 68L, 90L, 91L, 96L, 41L, 73L and 45L) representative isolates of the ribotype 027 subclades and nine other ribotypes (ribotypes 001, 220, 015, 002, 012, 020, 014, 005 and 078). Four other sequences including CD196, CDR20291 and QCD-66c26 (ribotype 027) and CD630 (ribotype 012) were obtained from *in silico* PCR. Other sequences were obtained from NCBI searches. Alignment was performed in MEGA5.01

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7.9 Appendix 9. Portal protein Sequences

C. difficile 027 portal protein sequences

>96L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>91L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>73L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>52L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>68L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVED

>45L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVED

>90L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>16L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>CD196

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>80L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>90L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>41L

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

>CDR20291

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDET

IVSNHPWVDD

Other *C. difficile* ribotypes and bacterial portal protein sequences

>**CD-001**

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKTVQITFE

>CD-002

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKTVQITF

>CD-014

ADVACSRPPWRPREFHRGVALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISDSKSYDYKTVQITF

>CD-015

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYDYKTVQITFR

>CD-020

ALKFLYSLLDLKCSKTEQKFKKAIRELLWFVCEYLKISGNKSYDYKTVQITF

>CD-078

ALKFFYRKLELKSGLLETEFRTSFDKLIKAILYFLGVTDYKKIQQTYTRNMMSNDLEDADIATKSVGIIPTKIILRHH

PWVDD

>CD-phage-phiCD27

ALKFLYSLLDLKCSKTEKKFKKAIRELLWFVCEYLKISGSKSYNYKSVQITFNHS

>CD-CF5

ALKFIYISETDLIKVQLKKESTFNLVKVIEHYILKHRPEKYKQGEEYYYGNTDVNNKRRYYLLDGAKVDDFTKVNNKA

INNYHKLLVDQKVGYSVGNPIVFNADDDNLTKLLNDLLGEEFDDTITELYLNASNKGVEWLHPYINRKGEFKYVIIPA

EEAIPIWDSKRQRELVAFIRFYYIEDIDGNKIKRVEYYTENDVTYFIERGNSFIQEFLYDEYGKMTDIQEGHFRINNK

EQGWGKVPFIPFKNNEKCVSDLTFYKSLIDIYDNNISTLADNLDEMQEVIYVLKEYPGTSLQEFIDNIRYYKSIKVDG

GGGVDKLEINIPVEAKKELLDRLEKNIIIFGQGVNPESQNTGDKSGVALKFLYSLLDLKCSKTEKKFKKAIRELLWFV

CEYLKISGSKSYDYKSVQITFNHSMIINESEKIDMASKSIGIISDETIVSNHPWVEDVNDELERLKKQEETQKEYDDL

IPTNNQEGVIDET

>**CD-QCD-63q42**

ALKNIYISETDLIKAQLKKESTFNLAKVIEHYILKHRPEKYQEGEAYYYGNANIKNKRRYYILDGAKVDDFTKVNNKA

VNNYHKLLVDQKVGYSVGKPIVFNADDNNFTKLLNELLGEEFDDTITELYLNASNKGIEWLHPYINRKGEFKYVIIPA

EEAIPIWDNKRQNEVVAFIRFYFIEDIDGNKIKRVEYYTENEVIYFIEKGNSFVQELLYDEYGKLTEIQEGHFRVNNK

EQGWGKVPFIPFKNNEKSVSDLTFYKSLIDIYDNNISTLADNLDEVQEAMYILKEYGGTDLVEFANNIRYYKAIKVGA

NGGVDKLEINIPVEAKKELLNRLEKNIIIFGQGVNPESQNTGDKSGVALKFLYSLLDLKCSKTEKKFKKAIRELLWFV

CEYLKISGSKSYDYKTVQITFNHSMIINEAEKIDMAAKSTGIVSDETIVSNHPWVEDV

>**CD-630**

ALKNIYISETDLIKVQLKKESTFNLVKVIEHYILKHRPEKYKQGEEYYYGNTDVNNKRRYYLLDGAKVDDFTKVNNKA

INNYHKLLVDQKVGYSVGNPIVFNADDDNLTKLLNDLLGEEFDDTITELYLNASNKGVEWLHPYINRKGEFKYVIIPA

EEAIPIWDSKRQRELVAFIRFYYIEDIDGNKIKRVEYYTENDVTYFVERGNGFVQEFLYDEYGKMTDIQEGHFRINNK

EQGWGKVPFIPFKNNEKCVSDLTFYKSLIDIYDNNISTLADNLDEIQEVIYVLKEYPGTSLQEFIDNIRYYKSIKVDG

GGGVDKLEINIPVEAKKELLDRLEKNIIIFGQGVNPESQNTGDKSGVALKFLYSLLDLKCSKTEKKFKKAIRELLWFV

CEYLKISGSKSYDYKTVQITFNHSMIINEAEKIDMAAKSTGIVSDETIVSNHPWVEDVNDELERLKKQEDTQKEYDDL

IPNNQDGVIDET

>**CD-phi-CD119**

AELEKIRAIISADAARRQEILQAKSYYYNKNDILKKGVVVQNRDENPLRNADNRISHNFHEILVDEKASYMFTYPVLF

DIDNNKELNEKVTDVLGNEFTRKAKNLAIEASNCGSAWLHYWIDEEYSGEQVTNQTFKYGVVNTEEIIPIYRNGIERE

LEAVIRYYIQLEDVKGQIQKQAYTYVEFWTDKILDKYKFFGVSCCGSQIEHITVQHRFNSVPFVEFSNNIKKQSDLSK

YKKILDLYDRVMSGFANDLEDIQQIIYILENFGGEDTSEFLKELKRYKTIKTETDSEGDSGGLKTMQIEIPTEARKII

LEILKKQIYESGQGLQQDTENFGNASGVALKFFYRKLELKSGLLETEFRTSFDKLIKAILYFLGVTDYKKIQQTYTRN

MMSNDLEDADIATKSVGIIPTKIILRHHPWVDDVEEAEKLYLEEKKIQASKVSDDYNNFTE

>**CD-phiC2**

ATDIQEGHFRINNKEQGWGKVPFIPFKNNEKCVSDLTFYKSLIDIYDNNISTLADNLDEIQEVIYVLKEYPGTSLQEF

IDNIRYYKSIKVDGGGGVDKLEINIPVEAKKELLDRLEKNIIIFGQGVNPESQNTGDKSGVALKFLYSLLDLKCSKTE

KKFKKAIRELLWFVCEYLKISGNKSYDYKTVQITFNHSMIINEAEKIDMAAKSTGIVSDETIVSNHPWVEDVNDELER

LKKQEDTQKEYDDLIPNNQDGVIDET

>*Clostridium-perfringens*-JS1495

MLLNLFNFRNFKDLFRNDINMMTVEEILYNEIKEFQASDRRAWMVIGDRYYRCENDILNRRIVRHTESGDIEDRSKAN

NRLAHGFVKNLVDEKIGYLLTKDYSLKCDNKEYIEKVKNVLGKYFQYTLTRLGYEASNKGIAWLQVYINEQGKFGMMI

IPAEQCVPLWKDNTHTELYGMIRYYVQTVYEGKEKKQITRVEYYTDKEVYFYVLDNDHLIPDIEQYEGGPILHYKKGE

EGRSWGKVPFIAWKNNHLEYPDVKFIKSLVDAYDKSRSEIDNFIEETKNLIYVLKGYGGENLSDFMKDLNYYRAIKID

DPEHGGVDTLTPKIDIQAAKEHFEQLKRDINEFGQGVPKDLDKYGNSPSGTALKFLYSGLDLKCNHLEVEFRQSFNQL

233

LYFVNRYLAENGQGNYENENVELIFNRDIQINETETINNCVNSKGIISDETILANHPWVSDVEEELKQIEKERKSEEP

PMFGEGDEE

>*Clostridium-perfringens*-JGS1987

MFGLGKPVEVEGPMNTIQWLGEEISDFLSSENRKQMLTGEQYYEVNNDIFQRKITRPTKKGGNEELKYKANNKLAHAF

YKNLVDEKVNYLLGKDYTLKSDNEEYIKKLDWTLGDDFLDILNELGYEASNKGIGWLHIFINEDSKLDYMVIPSEQII

PIWRDRRHKTLDRLIRIYDLLVYEGLEKKTVTKIELWYKDRVEYYIKDGELILLDSEKYLNVEGDIGHYLKDGEYAVW

GKIPFIAFKNNRIEKGDIKFIKSLIDNYDLSRSDVANFIEEVKNLIFVLKGYDGEDLGKFMDNLNYYRAIVLDDDGEA

STLNPTMDVEAIKIHYEQLKRDINECGQGVNKDLDKFGSAPSGIALKFLYSGIDLKCNSLEVLFKKAFKELLYFINIY

LSESSQGSYKDVPVELIFNKNIKINETETITNCINSKGVISNKTIMANHPWVQDPVAEQEQLDKEKEEFGINFDNIPL

PNKSIGENNEE

>*Bacillus-cereus*-phage

MIFIDKILNSGSNSVMTTEEIIQEEIKEWNSSQTRQLMLDGERYYKGDTDILKRKRMAIGEDGELEEVKNLANNKLVH

QFIRKLSDQKVGYLLSKPLSVQTDNEAYKNVLDDIFNKSFMRLLKNLGKDAINKGIAWAQIYYNEDGELRFKRLPSEE

IIPLWKDKEHTKLDALIRVYEVIVYEGKTKKTVQKVEYWDTKQVLRYINDNGKLITDVEAPEDEGHFSMVDDKGNKQS

FTWSKVPFVYFKYNDEEQPLIKFVKSLVDDYDRNKSDNSNNLEDLPNSIYVLKDYDGENLGEFRRNMSLYRAVKVAGD

GGVETRNLEINVEAYKTHIEQTRKDIYEFGRGVDTQSDKFGNSPSGIALKFLYNDLDMDCNIIETEFQASLEYLLWFV

NQHLINTGQDDFTNENVEFVFNRDTLINETDSINNCQNSVGIISDETIVANHPWATKDELEKIKKQKEERESMYPNFP

LEEIPEDEENEE

>Bacillus-phage-SPP1

MADIYPLGKTHTEELNEIIVESAKEIAEPDTTMIQKLIDEHNPEPLLKGVRYYMCENDIEKKRRTYYDAAGQQLVDDT

KTNNRTSHAWHKLFVDQKTQYLVGEPVTFTSDNKTLLEYVNELADDDFDDILNETVKNMSNKGIEYWHPFVDEEGEFD

YVIFPAEEMIVVYKDNTRRDILFALRYYSYKGIMGEETQKAELYTDTHVYYYEKIDGVYQMDYSYGENNPRPHMTKGG

QAIGWGRVPIIPFKNNEEMVSDLKFYKDLIDNYDSITSSTMDSFSDFQQIVYVLKNYDGENPKEFTANLRYHSVIKVS

GDGGVDTLRAEIPVDSAAKELERIQDELYKSAQAVDNSPETIGGGATGPALENLYALLDLKANMAERKIRAGLRLFFW

FFAEYLRNTGKGDFNPDKELTMTFTRTRIQNDSEIVQSLVQGVTGGIMSKETAVARNPFVQDPEEELARIEEEMNQYA

EMQGNLLDDEGGDDDLEEDDPNAGAAESGGAGQVS

>*E. coli*-phage-lambda

MAFRMSEQPRTIKIYNLLAGTNEFIGEGDAYIPPHTGLPANSTDIAPPDIPAGFVAVFNSDEASWHLVEDHRGKTVYD

VASGDALFISELGPLPENFTWLSPGGEYQKWNGTAWVKDTEAEKLFRIREAEETKKSLMQVASEHIAPLQDAADLEIA

TKEETSLLEAWKKYRVLLNRVDTSTAPDIEWPAVPVME

Figure 7.7 Multiple alignment of the portal gene sequences showing amino acids in region 1-121

The multiple alignment comprise of 29 amino acid sequences of 10 (96L, 52L, 68L, 45L, 90L, 73L, 91L, 41L and 16L) representative isolates of the ribotype 027 subclades and seven other ribotypes (ribotypes 001, 002, 015, 020, and 078). Two other sequences including ribotype 078 (M120) and ribotype 012 (CD630) were obtained from *in silico* PCR. Other sequences were obtained from NCBI searches. Sequence alignment was conducted in MEGA5.01
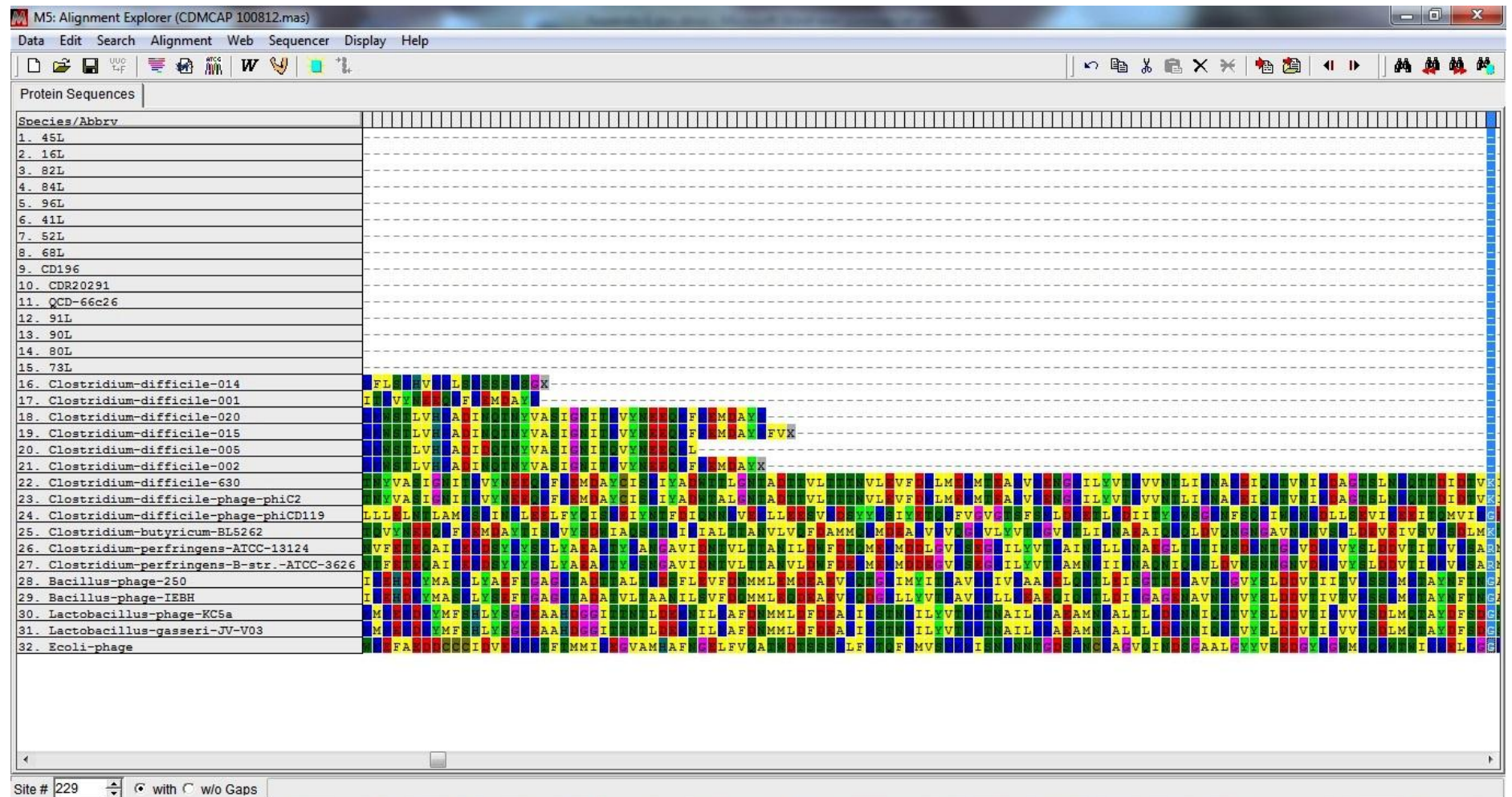
Figure 7.8 Multiple alignment of the portal gene sequences showing amino acids in region 121-241

Figure 7.9 Multiple alignment of the portal gene sequences showing amino acids in region 241-361

Figure 7.10 Multiple alignment of the portal gene sequences showing amino acids in region 361-481

Figure 7.11 Multiple alignment of the portal gene sequences showing amino acids in region 481-513 (from the blue line)

7.10 Appendix 10. Statistical Analysis of spore counts

All time point readings

Repeated Measures ANOVA

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	41
F	156.9
R squared	0.9874

Was the pairing significantly effective?

R squared	0.0002404
F	0.7641
P value	0.4691
P value summary	ns
Is there significant matching? (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	325.4	40	8.136
Individual (between rows)	0.07925	2	0.03962
Residual (random)	4.148	80	0.05186
Total	329.7	122	

48 h time point reading

Repeated Measures ANOVA

P value < 0.0001

P value summary   ***

Are means signif. different? $(P < 0.05)$ Yes

Number of groups   21

F   50.62

R squared   0.9620

Was the pairing significantly effective?

R squared   0.0007664

F   0.4036

P value   0.6706

P value summary   ns

Is there significant matching? $(P < 0.05)$ No

ANOVA Table	SS	df	MS
Treatment (between columns)	85.39	20	4.270
Individual (between rows)	0.06808	2	0.03404
Residual (random)	3.374	40	0.08434
Total	88.83	62	

72 and 96 h time points readings

Repeated Measures ANOVA

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	19
F	358.1
R squared	0.9944

Was the pairing significantly effective?

R squared	0.0002091
F	0.6778
P value	0.5141
P value summary	ns
Is there significant matching? (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	68.32	18	3.7
Individual (between rows)	0.01437	2	0.007184
Residual (random)	0.3815	36	0.01060
Total	68.71	56	

Pulsovar I

Repeated Measures ANOVA

P value	< 0.0001
P value summary	***
Are means signif. different? ($P < 0.05$)	Yes
Number of groups	16
F	209.5
R square	0.9905

Was the pairing significantly effective?

R square	0.001359
F	2.158
P value	0.1331
P value summary	ns
Is there significant matching? ($P < 0.05$)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	84.59	15	5.639
Individual (between rows)	0.1162	2	0.05809
Residual (random)	0.8075	30	0.02692
Total	85.51	47	

Pulsoavar IV

Repeated Measures ANOVA

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	23
F	134.3
R square	0.9853

Was the pairing significantly effective?

R square	0.0001656
F	0.2484
P value	0.7811
P value summary	ns
Is there significant matching? (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	217.3	22	9.878
Individual (between rows)	0.03653	2	0.01827
Residual (random)	3.235	44	0.07353
Total	220.6	68	

Pulsovars I and III

Repeated Measures ANOVA

P value	< 0.0001
P value summary	***
Are means signif. different? ($P < 0.05$)	Yes
Number of groups	17
F	217.3
R square	0.9909

Was the pairing significantly effective?

R square	0.0008816
F	1.548
P value	0.2282
P value summary	ns
Is there significant matching? ($P < 0.05$)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	94.62	16	5.914
Individual (between rows)	0.08427	2	0.04213
Residual (random)	0.8710 32	0.02722	
Total 95.58	50		

Pulsovars   III and IV

Repeated Measures ANOVA

P value   < 0.0001

P value summary   ***

Are means signif. different? (P < 0.05)   Yes

Number of groups   21

F   112.5

R square   0.9825

Was the pairing significantly effective?

R square   0.0001676

F   0.1920

P value   0.8261

P value summary   ns

Is there significant matching? (P < 0.05)   No

ANOVA Table	SS	df	MS
Treatment (between columns)	183.2	20	9.159
Individual (between rows)	0.03126	2	0.01563
Residual (random)	3.256	40	0.08140
Total 186.5 62			

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